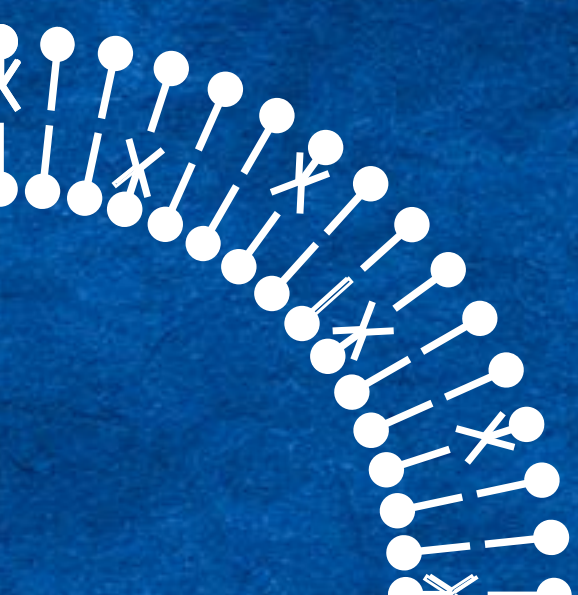
EXPANDING THROUGH COLLISIONS

A Dual Perspective on the Future of a Synthetic Cell

Coupling DNA and Tension-Mediated Vesicle Fusion for *in vitro* Membrane Growth

Building the Foundation of a Synthetic Cell Innovation Ecosystem



Tom F. Aarts

March 2022

Expanding Through Collisions

A Dual Perspective on the Future of a Synthetic Cell

-

Coupling DNA and Tension-Mediated Vesicle Fusion for *in vitro* Membrane Growth

Building the Foundation of a Synthetic Cell Innovation Ecosystem

by

Tom F. Aarts

To obtain the degrees of Master of Science
in Nanobiology and Science Communication
at Delft University of Technology

To be defended publicly on 11 March 2022

Student number	4452232
Project duration	8 February 2021 – 11 March 2022

Thesis committee Nanobiology

Prof. dr. G. Koenderink	Bionanoscience, Applied Sciences, TU Delft
Dr. C. Danelon	Bionanoscience, Applied Sciences, TU Delft
Dr. J. Zwanikken	Bionanoscience, Applied Sciences, TU Delft

Thesis committee Science Communication

Dr. É. Kalmár (First supervisor)	Science Education and Communication, Applied Sciences, TU Delft
Drs. C. Wehrmann (Second supervisor)	Science Education and Communication, Applied Sciences, TU Delft
Prof. dr. G. Koenderink (External supervisor)	Bionanoscience, Applied Sciences, TU Delft
Dr. M. van der Sanden	Science Education and Communication, Applied Sciences, TU Delft

TU Delft

Acknowledgements

First of all, I would like to thank Gijsje for being a supervisor for both projects of this thesis. I had a great time in your lab, enjoyed the scientific discussion we had, and appreciate how you enthusiastically took upon the role of external supervisor for my Science Communication project. I would also like to thank Lucia and Lennard for being the daily supervisors of my Nanobiology project. I was honoured to be part of team Membrane Expanders, and I learned a great deal from the discussions we had and the extensive feedback you gave me. Next to this, I look forward to what may stem from our project in the future. I would specifically like to thank Lucia for your suggestions on writing, your cookies during coffee breaks, and for sharing the joy of extrusion with me. I would like to thank Lennard for the large part of the data analysis you performed for the project, your fun stories about chickens, and for introducing me to the wondrous world of GUVs. I would also like to thank Iris, Nigel, Djim, and the other fellow ducklings for making life in the lab a lot more fun, especially in the company of Feel Good Radio. I would like to thank Éva for being the first supervisor of my Science Communication project. I enjoyed the fruitful discussions we had, and appreciate how you joined me in the exploration of a challenging case study. Next to this, I would like to thank Caroline for being the second supervisor of my Science Communication project, and the critical yet very useful feedback you gave me during our meetings. Furthermore, I would like to thank Jos Zwanikken and Christophe Danelon for being part of the Nanobiology thesis committee. I would also like to thank my housemates: Frits for cooking dinner for me while I was still in the microscopy room until 7 p.m., Nicky for being my fellow MEP buddy and for giving me tips on my figures, Sjors for providing me with a high-tech desktop set-up, Simone for coming up with the "Let's Make Vesicles" song, and Diedt for bringing some sjaarsch energy to the house. I would like to thank my family and friends for providing me with support and the necessary distraction throughout the project. Finally, I would like to thank Benjamin. Although I've asked for quite some patience from you over the last period, you continued to support me through thick and thin, and I can't wait to see how our next chapter will unfold.

Contents

List of abbreviations and glossary

Part I: Coupling DNA and Tension-Mediated Vesicle Fusion for *in vitro* Membrane Growth

cDICE	Continuous droplet interface crossing encapsulation
DLS	Dynamic light scattering
dsDNA	Double-stranded DNA
eDICE	Emulsion droplet interface crossing encapsulation
FACS	Fluorescence-activated cell sorting
FLIM	Fluorescence-lifetime imaging microscopy
FOV	Field of view
fps	Frames per second
FRAP	Fluorescence recovery after photo-bleaching
FRET	Fluorescence resonance energy transfer
Fusion rate	Fraction of fused vesicles in a vesicle population
FWHM	Full width at half maximum
GUV	Giant unilamellar vesicle
HPTS ratio	HPTS signal of GUV interior divided by HPTS signal of outer solution
LUV	Large unilamellar vesicle
MLV	Multilamellar vesicle
MPA	Micropipette aspiration
OBS	Observation buffer
PEG	Poly(ethylene) glycol
PSD	Particle size distribution
QCM-D	Quartz crystal microbalance with dissipation monitoring
RF	Radio frequency
SE	Spectroscopic ellipsometry

SLB	Supported lipid bi-layer
ssDNA	Single-stranded DNA
SUV	Small unilamellar vesicle
TEM	Transmission electron microscopy
w/o	Water-in-oil

Part II: Building the Foundation of a Synthetic Cell Innovation Ecosystem

Organisations and projects

AMC	Academic medical centre
BaSyC	Building a Synthetic Cell (Dutch fundamental research project)
BrisSynBio	"Multi-disciplinary research centre that focuses on the biomolecular design and engineering aspects of synthetic biology" [1]
EBRC	Engineering Biology Research Consortium
HGP	Human Genome Project
I&I Centre	Innovation & Impact Centre
NWO	Dutch Research Council (Nederlandse Organisatie voor Wetenschappelijk Onderzoek in Dutch)
OCW	Dutch Ministry of Education, Culture and Science (Onderwijs, Cultuur en Wetenschap in Dutch)
SynCellEU	The Synthetic Cell Initiative (European collaboration, aims to explore synthetic cell applications next to performing research)
Unicamp	University of Campinas

Innovation and collaboration models

FOMO	Fear of missing out (identified challenge of PACES model)
Linear innovation model	Innovation model where innovation is realised in a linear fashion from universities to firms (also technology-push model or "Mode 1")
Non-linear innovation model	Innovation model describing bilateral interactions between universities, university-related institutions and firms to realise innovation (also "Mode 2")
PACES model	"Preserving and cultivating effective suspense" model
Quadruple helix model	Collaboration model describing bilateral interactions between academia, industry, governmental organisations, and the public
Technology-push model	Innovation model where innovations are based on scientific findings rather than societal needs (also linear innovation model or "Mode 1")
Triple helix model	Collaboration model describing bilateral interactions between academia, industry, and governmental organisations

Business and innovation terms

Entrepreneurial capital	The capabilities and commitment of individuals or groups to perform entrepreneurial activities

Firm	For-profit business
Human capital	"The skills, knowledge, and experience possessed by an individual or population, viewed in terms of their value or cost to an organization or country." [2]
IP	Intellectual property
KPI	Key Performance Indicator (Indicators used to assess the performance of a company)
KTT	Knowledge and technology transfer
SME	Small and medium-sized enterprise
Social capital	"The networks of relationships among people who live and work in a particular society, enabling that society to function effectively" [3]
Supply chain	The chain of activities and processes required to develop and distribute a product
TRL	Technology Readiness Level
TTO	Technology transfer office
Value capture	Creating competitive advantage by maximising profits
Value chain	Activities and processes required to develop a product or to provide a service
Value creation	Creating value for consumers and other parties

Other

BioE	Bioentrepreneurship
CLab	Contamination lab
ELSI	Ethical, legal and societal implications
FET Flagships	Future and Emerging Technologies Flagships
PI	Principal investigator
PR	Public relations
PUS	Public understanding of science
RI	Responsible innovation
RRI	Responsible research and innovation

1 General introduction

How do you build your own cell? In 2017, a group of seventeen Dutch scientists embarked on the courageous mission to answer this question, with the goal to develop a synthetic cell within ten years [4]. The name of this collaborative research project is "Building a Synthetic Cell", or shortly BaSyC. The word "building" in the project's name illustrates its engineering approach: using the principles of physics, chemistry and biology, the researchers want to create a synthetic cell from scratch. This means that the synthetic cell will be build with a bottom-up approach: individual biomolecules will be used as building blocks to shape something that resembles a real cell.

The main motivation to build a synthetic cell is that many biological processes that are essential for life, like cell division [5], DNA folding [6], and cell-to-cell communication [7], are still not fully understood. One of the reasons that we still lack a full understanding of these processes is that many of them are occurring simultaneously within and around cells, mediated by millions of proteins and other biomolecules [8]. We basically cannot see the wood for the trees, or to take it literally, we cannot see the proteins for the cell. The philosophy is that by finding out what type of biomolecules are essential for a specific cellular process to occur, we will start to better understand it.

We can compare a cell with a large and busy city, where people transport in all sorts of different vehicles, where new buildings are constantly being constructed and broken down, and where information is spread through a variety of different channels. Similarly, in cells, proteins and other biomolecules are synthesised and transported in different ways, all having their own approach of forwarding messages and executing their tasks. Cellular structures provide support and are simultaneously integrated in complex decision making networks. Moreover, cells are resilient to uncertain events by constantly degrading and reassembling biomolecules.

The difference between a large city and cells is that cells are small, and we require high-technology equipment to observe the processes taking place in them. Another difference is that cities are built by men, while cells are created by nature. We have a general understanding of how cities work, since we have built them ourselves. Although we are all built up from cells, we do not exactly know how they work, collaborate with each other and make us alive. The idea of BaSyC is that by building a synthetic cell from its basic components, we will unravel the complexity of many biological processes. By integrating these individual processes into a synthetic cell, we will get a better grasp of life.

1.1 The goal and organisation of BaSyC

Reconstituting all cellular processes in a synthetic cell within ten years is considered to be an impossible task with the current existing technologies and available knowledge. The researchers of BaSyC therefore determined to focus on three essential processes for cellular life: the synthetic cell should be able to sustain itself with energy and nutrients through cell fuelling (Figure 1.1, green), it should be able to read off the genetic code by DNA processing (Figure 1.1, orange and red), and it should be able to split into two daughter cells through cell division (Figure 1.1, blue).

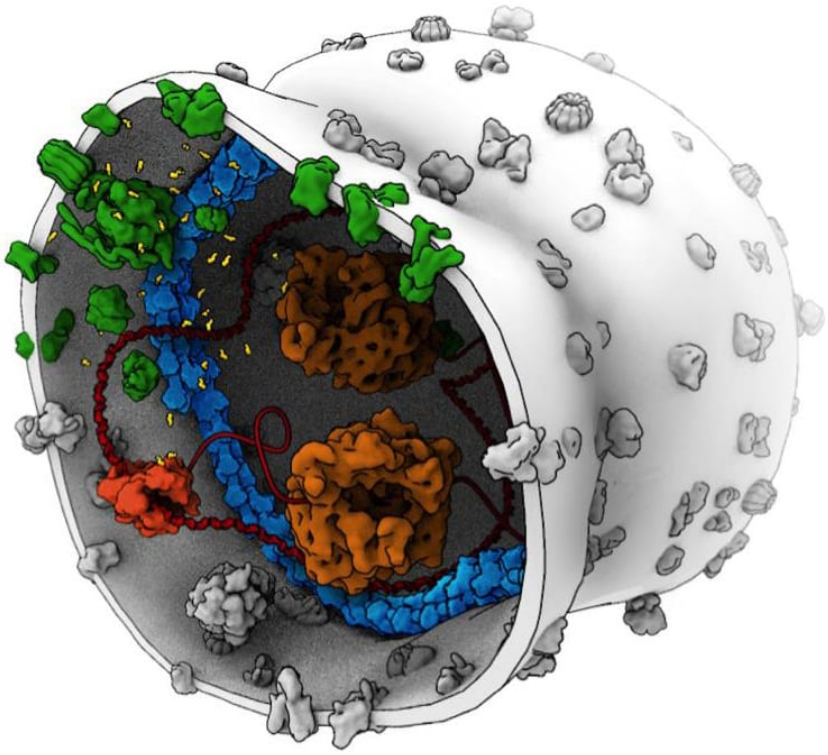


Figure 1.1 Artist's impression of what the synthetic cell could look like, including the three essential processes that BaSyC is focusing on. Cell fuelling is depicted by the green and yellow structures, representing membrane proteins and other molecules required for the energy metabolism of a cell. DNA processing is indicated by the orange molecules reading of the genetic code, which is displayed in red. The contractile ring, which is a large molecular structure required for cell division, is presented in blue. Picture made by Graham Johnson and retrieved from the BaSyC website [4].

The great complexity of a synthetic cell requires scientists from different disciplines to work together. These are not only scientists with a background in the natural sciences (i.e. mathematics, physics, chemistry and biology), but also researchers with a background in philosophy. Building a synthetic cell raises ethical and philosophical questions that are important to be addressed. Part of these questions are fundamental in nature, and are for example concerned with the definition of life, and how the development of a synthetic cell will bring us further. As the project proceeds and the technology is further developed, the questions become more concrete. These more applied questions for example ask whether a computational model is a suitable methodology to better understand life, and how society can be involved in BaSyC.

The organisational structure of BaSyC is visible in Figure 1.2. The research group leaders, or principal investigators (PIs), together form the Consortium Assembly. Together, this group of people works on the different work packages (WPs) that are part of the project. The first work package (WP0) is concerned with theoretical and computational models that aim to capture the complexity of biological systems. The feedback from experimental studies is constantly integrated into these models. These experimental studies are performed in the next three work packages (WP1-3), that cover the three cellular processes found to be essential for a synthetic cell as depicted in Figure 1.1. WP4 is concerned with the integration of the different cellular processes, or "modules" as they are called by the consortium, and WP5 is about the synthetic cell becoming autonomous, being independent of any external support. Lastly, WP6 is concerned with the philosophical, ethical and societal aspects of a synthetic cell. To study these aspects with people not only from BaSyC itself, a Future Panel consisting of natural and social scientists, policymakers, media experts and artists has been set up [9] (not included in Figure 1.2).

The final responsibility of the project lies with the Steering Committee, a group of six researchers chaired by prof. dr. Marileen Dogterom, who is a professor in biophysics and bionanoscience at Delft University of Technology. The Steering Committee oversees that the different work packages are integrated in time, and determines together with the Consortium Assembly how grants are divided over the programme. They receive assistance from the Programme Manager and Support Office, who monitor the project's status and daily tasks. The Programme Manager and Support Office also organise BaSyC events and educational and outreach activities. The steering Committee itself is monitored by the International Advisory Board, consisting of renowned scientists from Europe and the United States. They provide advice both on a scientific and a strategic level.

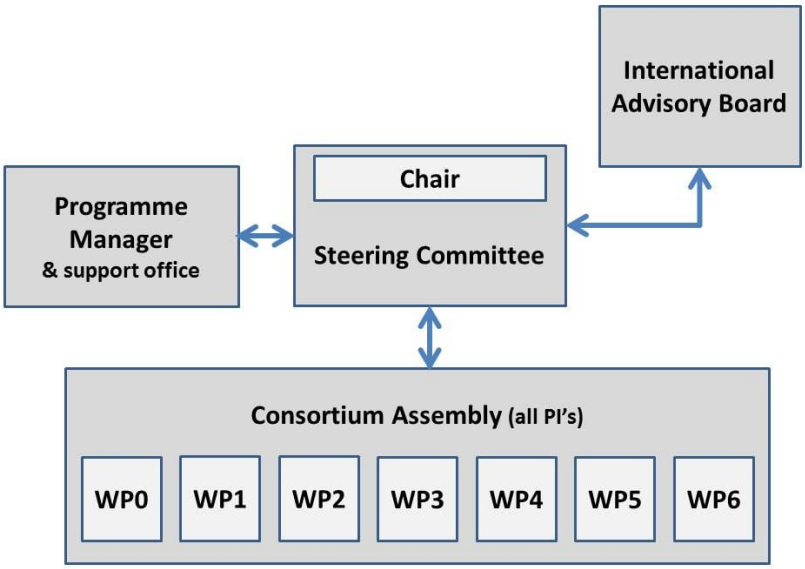


Figure 1.2 The organisational structure of BaSyC. Figure retrieved from the BaSyC website [4].

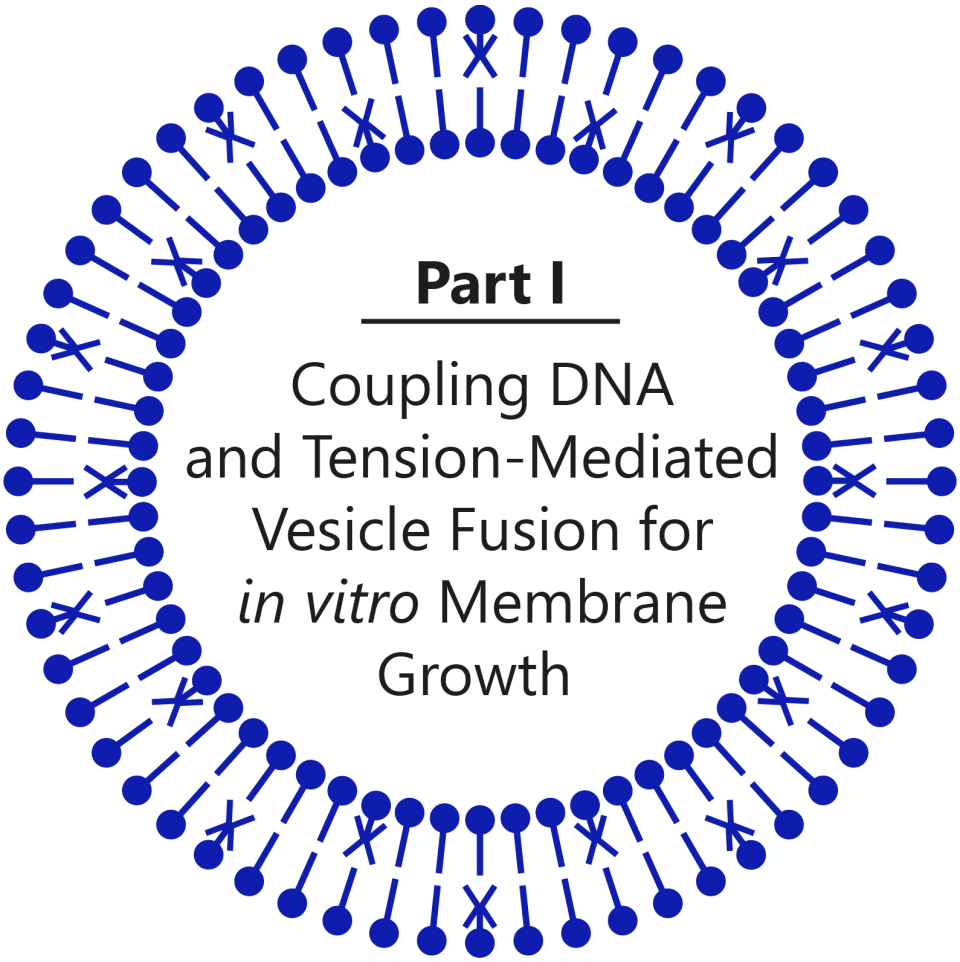
1.2 Thesis structure

In this thesis, we will describe two studies performed in the context of a synthetic cell. The research for the Nanobiology part of this thesis (Part I) was performed in the group of prof. dr. Gijsje Koenderink, who was part of the Steering Committee of BaSyC when the project started in 2017. This research group studies the mechanics of cells, and how cells can be strong yet flexible at the same time. The group of Koenderink specifically focuses on the cytoskeleton, which can be considered as the bones and muscles of a cell. Next to this, the cytoskeleton has an important function in cell division, which is one of the modules BaSyC aims to reconstitute in a synthetic cell. The Nanobiology part of this thesis will focus on synthetic cell division, and specifically on the acquirement of additional membrane area that is required for this process. Here, we adopted a strategy for membrane growth based on the fusion of differently sized vesicles, where larger vesicles served as a model for cells, whereas smaller vesicles served as membrane donors. To facilitate membrane growth, we explored the parameter spaces of DNA and tension-mediated vesicle fusion. A systematic study on the combination of these two fusion strategies had not been performed before, and we postulate that it serves as a promising route for sustained synthetic cell division.

Next to obtaining a better understanding of life, synthetic cell research can lead to revolutionary applications in the future. Possible applications that have been suggested are the development of new drugs [10], drug delivery systems, and sustainable energy solutions [9]. BaSyC is funded through the Gravitation programme of the Dutch Ministry of Education, Culture and Science (OCW), for which the Dutch Research Council (NWO) makes the selection. These grants are specifically intended for research programmes that have the potential to become world leading in their field. The funding of the NWO promotes fundamental research, and BaSyC is therefore less involved in exploring the potential

future applications of synthetic cells. A collaboration that is more concerned with this is the Synthetic Cell Initiative (SynCellEU). SynCellEU is a collaboration between BaSyC and other European synthetic cell hubs. This collaboration currently consists mainly of researchers, but it has the long term vision to become an innovation ecosystem, where scientists will actively collaborate with other actors like industry and society. The Science Communication part of this thesis (Part II) will explore how the foundation of such an innovation ecosystem can be built, especially focusing on the collaborations between academia and industry. Next to this, it will focus on the implementation of responsible research and innovation (RRI) elements, thereby ensuring a sustainable innovation ecosystem.

The two studies will explore future expansions of a synthetic cell with a dual perspective. In both cases, these expansions will be facilitated through collisions. In the Nanobiology project, we will show that collisions between differently sized vesicles allow these vesicles to fuse, thereby achieving membrane growth that is required for a synthetic cell to divide. In the Science Communication project, we will present the essential role of collisions between different partners for a synthetic cell innovation ecosystem to thrive. In the final part of the thesis, the two projects will be combined into a general discussion, reflecting on the future of a synthetic cell (Chapter 17). Together, the two studies will present potential future scenarios of a synthetic cell, that can both be used to realise this revolutionary technology and to develop impactful applications from it.



Part I

Coupling DNA and Tension-Mediated Vesicle Fusion for *in vitro* Membrane Growth

2
Background

2.1 The requirement of membrane growth for synthetic cell division

In order to better understand the molecular basis of life, researchers are aiming to mimic essential biological processes such as energy metabolism, DNA replication, and cell division [11]. Successful attempts to reconstitute these cellular modules with a bottom-up approach are emerging over the last couple of years [12]. By combined reconstitution of all these vital processes into a single system, scientists are attempting to build a synthetic cell [9].

Cell division is one of the key processes of life. There are various ways to reconstitute cell division, inspired by different mechanisms occurring in vivo or by engineering approaches. These for example comprise approaches based on the reconstitution of a minimal bacterial divisome [13], the induction of spontaneous curvature by membrane-bound proteins [14], the application of shear forces, [15], or the sole presence of an osmotic gradient [16]. Synthetic cell division does not only involve membrane fission, but also requires membrane growth. Cellular volume is conserved during cytokinesis in living cells. Since the surface-to-volume ratio of the daughter cells is higher compared to the mother cell, an increase in membrane area of 26% is required to allow for cell division [17]. In this study, we aim to generate membrane growth *in vitro* to enable synthetic cell division.

2.2 *In vivo* membrane growth

In living animal cells, membrane trafficking is found to be essential for obtaining extra membrane area prior to cytokinesis [5]. This process involves the intracellular exchange of small vesicles through endo- and exocytosis [18, 19]. McCusker and Kellogg described how vesicles transported from the Golgi apparatus to the plasma membrane are involved in membrane growth [5]. The model by Fürthauer and González-Gaitán shows how cells in interphase continuously internalise and recycle endosomes storing membrane area with equal rates, resulting in a constant cell surface area (Figure 2.1a). Prior to cell division, however, cells stop to recycle endosomal compartments (Figure 2.1b), thereby decreasing cell surface area and obtaining a more spherical shape (Figure 2.1b'). When cells start to divide during the process of cytokinesis, stored membrane area is extensively recycled to acquire the cell surface area required for division (Figure 2.1c).

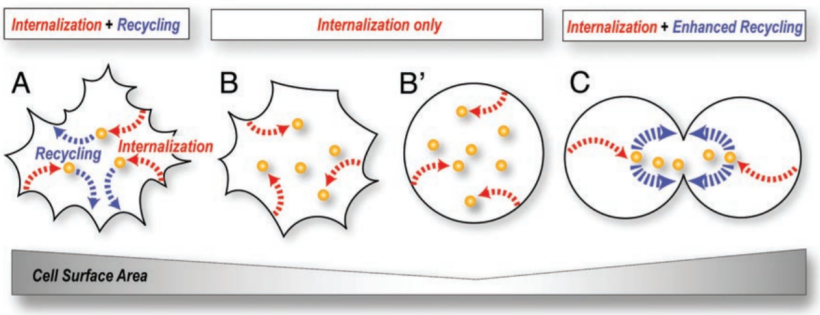


Figure 2.1 Model describing membrane growth during cytokinesis *in vivo*. Membrane area is continuously internalised and recycled during interphase (a), only internalised prior to cell division (b) resulting in a more spherical cell shape (b'), and extensively recycled during cytokinesis (c). Figure retrieved from Fürthauer and González-Gaitá [18].

2.3 Vesicles as a model for a synthetic cell and cellular compartments

The synthetic cell relies on the encapsulation of specific biomolecules in a semi-permeable compartment, thereby separating its interior from the external environment. Containers used to form this compartment can be sorted in three categories: membranous structures (vesicles, emulsions, and coacervates) [20]. Here, the latter two are containers where the separation between the internal and external environment is facilitated through liquid-liquid phase separation, either by an water-in-oil emulsion or by spontaneous demixing. Membranous structures, in turn, can be sub-divided into liposomes, vesicles formed by fatty acids, and polymersomes.

The building blocks of liposomes are natural phospholipids. Due to the amphiphilic character of phospholipids, they can self-assemble into vesicles consisting of a single mono-layer, called micelles, or form vesicles built up from one or multiple bi-layers, termed unilamellar or multilamellar vesicles, respectively [21]. Similar to living cells, liposomes are vesicles consisting of a single lipid bi-layer (Figure 2.2a), giving them close resemblance to real cell membranes. In the context of a synthetic cell, liposomes are therefore studied most extensively [20]. Liposomes are called giant unilamellar vesicles (GUVs) when sized > 1 µm, making them a good model for eukaryotic cells [22].

To model extracellular vesicles or cellular compartments, such as endosomes and lysosomes, smaller vesicles are more useful. Here, we distinguish large unilamellar vesicles (LUVs) with a diameter from 100 nm to 1 µm, and small unilamellar vesicles (SUVs) with a diameter below 100 nm [23]. Since the volume of a sphere is proportional to the cube of its radius, the volumes of the individual vesicle types differ dramatically, as becomes apparent from the schematic representation in Figure 2.2b. Here, the sizes of the vesicle types are relatively scaled, causing the LUV and SUV to be barely or not visible. There is another striking difference in size between the vesicle membrane and vesicle interior. A membrane consisting out of POPC lipids is only 4 nm thick. When scaling a 50 µm sized GUV to a balloon of 50 m, this would mean that the shell of the balloon would be only 4 mm thick [21]. This size difference illustrates that membranes should have a very stable structure, while at the same time allowing for the passage of small molecules through diffusion and osmosis.

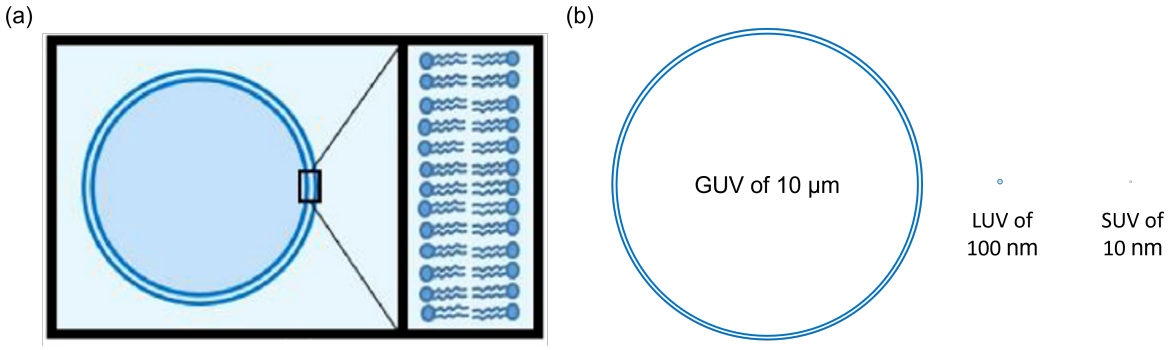


Figure 2.2 (a) Schematic representation of a liposome consisting of a single lipid bi-layer. Figure adapted from Spoelstra et al. [20]. (b) Scaled representation of a GUV, LUV and SUV, where the LUV and SUV are barely or not visible. Figure adapted from Walde et al. [21].

Liposomes are thus extensively described in literature, exist in different sizes, and their membranes closely mimic those of real cells. We will therefore use liposomes to study *in vitro* membrane growth in this project. More specifically, we will study fusion between LUVs and GUVs, where GUVs will serve as a model for cells, and LUVs will act as membrane donors. Since LUVs have a higher surface-to-volume ratio than GUVs, fusion will result in an increase of surface-to-volume ratio of the GUV. In this way, it relates to the process of cytokinesis, where cellular volume is kept constant during cell division (Section 2.1). The dramatic size difference between GUVs and LUVs also means that we require a large number of fusion events to obtain significant membrane growth. We will therefore focus on current approaches to maximise fusion efficiency in Sections 2.7, 2.8, and 2.9.

2.4 Vesicle formation methods

There are numerous methods to form vesicles. To produce GUVs, the simplest approach is probably through spontaneous swelling of dried lipid films (Figure 2.3a). In this method, that was originally developed by Reeves and Dowben [24], layers of lipids are dried on a solid surface. After the addition of aqueous buffer, hydration of the lipid head groups causes the dried films to swell. Energetically unfavourable interactions between water and the lipid tails consequently result in closure of the vesicles. There are various modifications to this original swelling method, involving either promotion of swelling by the application of an electric field (electroformation) [25] or by swelling on top of a porous hydrogel [26].

While swelling methods allow formation of a wide range of membranes in different buffers, they are limited with respect to encapsulation of solutes, which is typically required for more complex reconstitution assays. For these assays, emulsion-based methods are more useful. In these methods, the head groups of lipids in oil adsorb to water-in-oil (w/o) emulsions, thereby creating lipid mono-layers. A second mono-layer is subsequently formed by passage of the w/o emulsions through an oil-water interface [27] (Figure 2.3b). Here, the w/o emulsions are for example prepared using a capillary (as in the continuous Droplet Interface Crossing Encapsulation (cDICE) method [28]) or based on coarse mechanical agitation (as in the emulsion Droplet Interface Crossing Encapsulation (eDICE) method [29]). The w/o emulsions can subsequently be transferred through the oil-water interface via gravity [27], centrifugal forces [28], or microfluidic channels [30]. GUV formation methods based on oil-water crossings allow for better control over vesicle size and encapsulation efficiency compared to swelling methods [31]. There are also some downsides to w/o emulsion transfer methods: oil remnants can be present in the formed membranes, and they are generally more time and labour-intensive than swelling methods.

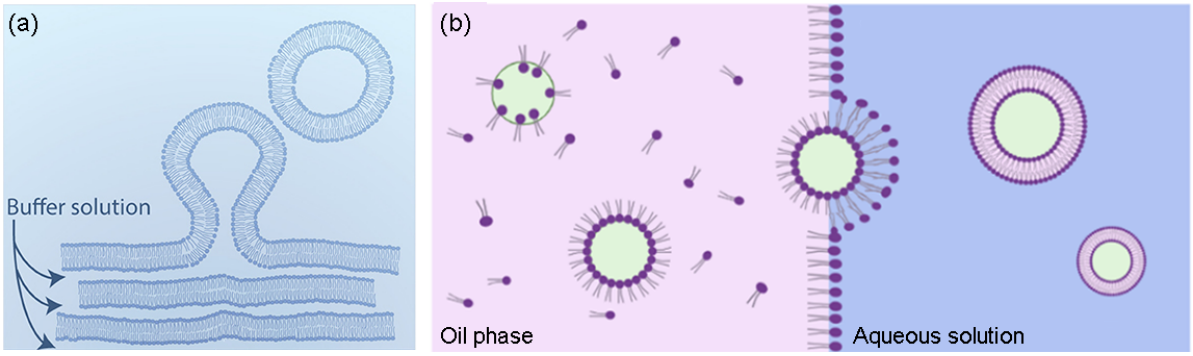


Figure 2.3 Vesicle formation methods typically used to prepare GUVs. (a) Adding aqueous buffer to hydrated lipid films leads to the spontaneous swelling of vesicles. Figure adapted from Stein et al. [32]. (b) Passage of w/o emulsions through an oil-water interface leads to the formation of vesicles. Figure adapted from Lambert [29].

Similar to the spontaneous swelling method, LUVs and SUVs are typically formed by the hydration of lipid films [33]. Contrary to GUV formation, however, the lipid films are shaken, vortexed, or sonicated after addition of the aqueous buffer, to yield multilamellar vesicles (MLVs). LUVs and SUVs can subsequently be formed from these MLVs, where lipid layers of these onion-like vesicles are peeled off through the supply of external energy [21]. This energy is typically supplied through ultrasonication, french press extrusion, or extrusion through porous membranes [33]. Here, vesicle size can be tweaked through input power, process time, or pore size.

2.5 *In vitro* membrane growth

Scientists are undertaking a variety of approaches to achieve *in vitro* growth of a synthetic cell membrane. Some of these approaches are based on the synthesis of phospholipids through an encapsulated minimal genome [11]. One of the main advantages of these approaches is that they would allow a synthetic cell to be self-sustainable. Blanken et al. showcased the successful synthesis and incorporation of phospholipids in a liposomal membrane [34]. However, a large heterogeneity in the amount

of phospholipids produced among vesicles was observed, with two different lipid species resulting in no lipid enrichment for about 50 or 90% of liposomes. Moreover, the design and encapsulation of a minimal genome is a complex process, and with a required overnight incubation step, the timescale to acquire fusion is relatively long.

Having a shorter fusion timescale would be advantageous for methodological efficiency. Possibly simpler approaches to achieve membrane growth are based on the fusion of smaller vesicles with a larger vesicle, similar to the model depicted in Figure 2.1. A difference with the presented model, however, is that small vesicles are typically added externally in reconstitution experiments. Another difference is that in living cells, vesicle fusion is mediated by SNARE proteins [18] (see Section 2.9), whereas in reconstitution experiments, scientists often make use of simple physical principles. Examples of these physical principles are the release of membrane tension, the attraction of opposite membrane charges, and DNA hybridisation, which will be discussed in Sections 2.7, 2.8, and 2.9, respectively.

Another interesting approach for membrane growth is the spontaneous fusion of fatty acid vesicles with fatty acid micelles. In earlier work, the surface area of a single vesicle could be increased by ~3.7-fold with this approach [15]. However, fatty acid-based vesicles are less interesting in the context of a synthetic cell, since they are unstable in the presence of divalent cations, block polymerase activity, and require non-polar conditions unlike most current life forms [11]. In the next section, we will describe the different options for a synthetic cell container, and we will present what type of vesicles we will use in this study.

2.6 Mechanics of membrane fusion

Before we will dive into possible strategies for GUV-LUV fusion, we will first describe some of the mechanics involved in membrane fusion. Insights in the mechanics of membrane fusion are largely based on theoretical models [35]. Here, it is generally accepted that membranes in proximity can form an intermediate hemifusion stalk (Figure 2.4a.iii), followed by the opening of a pore to complete fusion (Figure 2.4a.v). Experimental research has confirmed the existence of the hemifusion stalk, where only the outer leaflets of the fusing vesicles are locally merged [36]. In this intermediate state, lipid mixing can occur in the outer leaflets, but inner content mixing of the two adjacent vesicles is not possible. More recently, the hemifusion diaphragm (Figure 2.4a.iv) was visualised in an experimental setting [37]. The formation of the point-like-protrusion, (Figure 2.4a.ii), on the other hand, is only predicted in the membrane fusion model developed by Efrat et al. [38] and lacks experimental evidence.

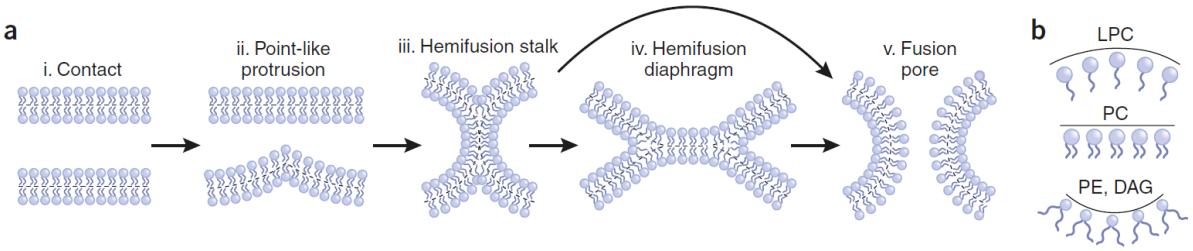


Figure 2.4 (a) Initial (i), intermediate (ii-iv) and final (v) states of membrane fusion. Formation of the hemifusion stalk (iii) and hemifusion diaphragm (iv) are confirmed experimentally, whereas the existence of the point-like protrusion (ii) is only predicted in the model developed by Efrat et al. [38]. (b) Lipid composition has an effect on fusion state formation rates, which is thought to be due to the generation of spontaneous curvature. Mono-layers consisting of cylindrical lipids (PC) have no spontaneous curvature. Mono-layers of inverted cone-shaped lipids (LPC) generate a positive spontaneous curvature, whereas mono-layers of cone-shaped lipids (PE and DAG) generate a negative spontaneous curvature. Figure adapted from Chernomordik and Kozlov [35].

In earlier work, it was found that lipid composition influences the formation rate of specific fusion states. Chernomordik et al. described that cone-shaped lipids (PE and DAG in Figure 2.4b) promote the formation of the hemifusion stalk, whereas they inhibit pore formation [39]. Inverted cone-shaped lipids (LPC in Figure 2.4b), on the other hand, inhibit the formation of the hemifusion stalk, but promote pore formation. Next to this, cone-shaped lipids generate a negative spontaneous curvature in lipid mono-layers, whereas inverted cone-shaped lipids generate a positive spontaneous curvature (Figure 2.4b).

From these observations, Chernomordik and Kozlov concluded that negative curvature is required to form the hemifusion stalk (corresponding to the membrane kink of the point-like protrusion), and that positive curvature is required to form the fusion pore (corresponding to the "net positive curvature of the pore edge") [35]. Other work based on molecular-dynamics simulations showed that the presence of cone-shaped PE lipids can also promote the formation of the fusion pore, but that the PE content should be properly balanced [40].

Theoretically, the point-like protrusion decreases the energy of hydration repulsion between two apposing membranes [35]. More recent theoretical work by Akimov et al., however, states that the formation of the point-like protrusion is physically impossible [41]. The theoretical fusion state requires a force from the point-like protrusion perpendicular to the membrane, but according to the authors, "during "normal" fusion, no possible source of such force can be found" [41]. Instead, they present the hydrophobic defect as an intermediate state between unfused and hemifused membranes, where small openings are created in the fusing membranes (Figure 2.5). Here, it is assumed that the energy cost of exposing part of the hydrophobic core to water is lower than the energy associated with hydration repulsion. Similar to the point-like protrusion, the formation of the hydrophobic defect state requires the generation of negative curvature. However, in case of the hydrophobic defect, negative curvature should be generated in both of the fusing membranes.

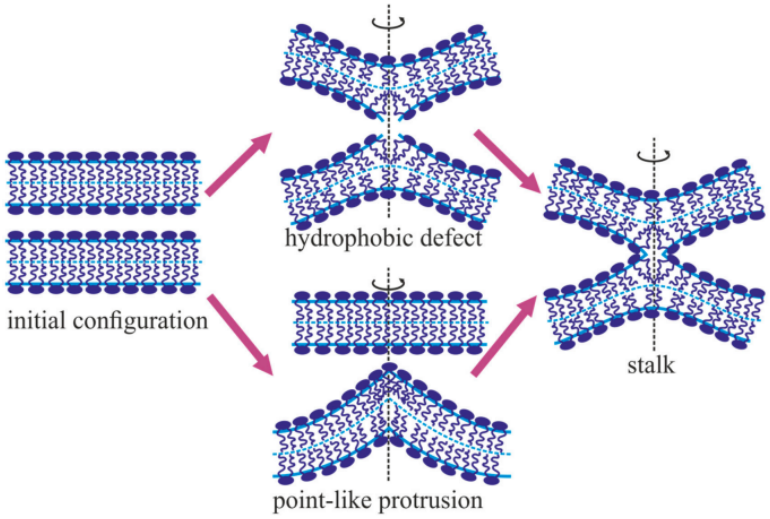


Figure 2.5 Proposal of the hydrophobic defect as the intermediate state between unfused and hemifused membranes instead of the point-like protrusion. Figure retrieved from Akimov et al. [41].

Apart from lipid composition, it has been shown that vesicle size has an effect on fusion efficiency. Here, it was found that smaller vesicles are more fusogenic [e.g. 42, 43], which is thought to be due to an increased positive curvature for smaller vesicles [44]. This corresponds with the correlation between positive curvature and fusion pore formation described by Chernomordik and Kozlov [35]. Higher curvature is also associated with increased membrane tension [44], suggesting that fusion could perhaps be promoted by inducing membrane tension [30]. We will elaborate on this potential strategy for membrane growth in the next section.

2.7 Tension-mediated membrane fusion

In the 1970s, it was already found that the swelling of small vesicles results in an increased exocytosis rate in unicellular organisms [45], a process that requires membranes to fuse. Later work showed that exocytosis is also promoted when cell membranes are put under tension [46]. Through the uptake of membrane area during exocytosis, cells are able to release their tension. These findings correspond with the model depicted in Figure 2.1, where increased membrane recycling is associated with an increase in cell surface area during cytokinesis [18]. Endocytosis, on the other hand, is inhibited when cells are tense, possibly due to membrane tension forces counteracting the force required to deform

the membrane during endocytosis [47].

Based on the observations of tension-mediated vesicle fusion in living cells, scientists have made the first attempts to reconstitute the process *in vitro*. Most notable of these studies, and mostly related to this research project, is the work by Deshpande et al. [30]. They successfully showed the growth of GUVs by fusing them with 30 nm SUVs. Here, the GUVs were put under tension by placing them in a hypotonic solution, thereby swelling the GUVs through the influx of water. After an overnight incubation, membrane growth was achieved for 15-25% of the GUVs. Based on a shift in GUV size distributions, the authors estimated that a small fraction of ~2% of the GUVs even doubled their volume. The absence of membrane growth for the other vesicles is attributed to rupture-reseal events, where the membrane temporarily forms a pore, thereby releasing osmotic stress. Theoretically, the method developed by Deshpande et al. could also lead to GUV-GUV fusion events, but these were not observed in the experiments.

Arribas Perez and Beales combined the tension-mediated approach with a fusion strategy based on silica nanoparticles [48]. Since nanoparticles are able to deform and remodel membranes, they can be used to enhance and control membrane fusion. Arribas Perez and Beales showed that ~32% of tense GUVs exposed to nanoparticles underwent fusion with another GUV, compared to ~16% of relaxed GUVs.[1] Here, relaxed GUVs were placed in a hypertonic solution, causing the vesicles to osmotically deflate. For LUV-LUV fusion (d = 400 nm), a lipid mixing rate of ~80% was found for tense LUVs, compared to ~60% for relaxed LUVs. These rates indicate the fraction of vesicles within a sample that exhibited lipid mixing. Lipid mixing could have occurred for vesicles that did not fully fuse but were trapped in a hemifused state (Section 2.6). However, the fractions of fully fused vesicles (i.e. the fusion rates) were determined with dynamic light scattering (DLS) measurements, and these values corresponded to the values found for lipid mixing. Since Arribas Perez and Beales found higher fusion rates for 400 nm LUVs compared to GUVs [48], their results correspond to the finding that smaller vesicles are more fusogenic (Section 2.6).

Despite the successful results in tension-mediated membrane fusion, literature is not consistent on this phenomenon. By studying SUV-SUV fusion (d = 26 nm) and LUV-LUV fusion (d = 120 nm), Malinin et al. found an opposite effect, where fusion was promoted for relaxed vesicles instead of tense vesicles [42]. This effect was especially apparent for SUVs, where almost 50% of relaxed vesicles showed content mixing, compared to practically no fusion for tense vesicles. The authors state that vesicle relaxation can be beneficial to move lipids into hydrophobic interstices, structures associated with the hemifusion diaphragm depicted in Figure 2.4a.iv. By promoting the formation of this final intermediate fusion state, the authors postulate that full fusion is enhanced as well. However, the existence of hydrophobic interstices is contested [41]. Another possible explanation for the contradicting results presented by Malinin et al. is that they induce fusion with poly(ethylene glycol) (PEG) polymers. This compound is known to have many effects on membranes: it increases phase transition temperatures [49], causes lipid dehydration [50], and promotes membrane porosity [51]. Possibly, these PEG-membrane interactions caused the contradicting results, for example by the release of osmotic tension due to membrane porosity. It should also be noted that fusion is dependent on vesicle size (Section 2.6) - SUV-SUV studies can therefore not be related directly to GUV-SUV studies.

Due to its relative simplicity, tension-mediated fusion is attractive to utilise for membrane growth. However, it might be beneficial to combine the method with another approach to lower the fusion timescale, further boost the fusion efficiency, and to gain a better control over the fusion process. Next to this, membrane growth is ideally facilitated through a strictly selective fusion mechanism that only allows GUVs to fuse with LUVs or SUVs. We therefore combined the tension-mediated approach with another strategy to enhance fusion. Here, we investigated if the two fusion methods were compatible with each other.

[1] Since only half of the fusion events could be detected with the method of Arribas Perez and Beales, these fusion rates are theoretical values [48].

2.8 Charge-mediated membrane fusion

In the previous section, we described that a selective fusion mechanism is preferred for *in vitro* membrane growth, i.e. a mechanism that only allows for fusion between GUVs and LUVs or SUVs. One way to achieve this is through charge-mediated fusion, where vesicles with opposite charges attract each other and can subsequently fuse. Vesicles can be endowed with a charge component by including oppositely charged phospholipids in their respective compositions. Lira et al. showed that negatively charged GUVs start to fluctuate upon successful fusion with positively charged LUVs, indicating membrane growth [52]. Next to this, they found that fusion efficiency increases when higher fractions of charged lipids are used. The same trend was observed for SUV-SUV fusion by Biner et al. [43].

Using higher fractions of charged lipids, however, comes at a cost: it decreases the ability of vesicles to reseal membrane pores; a process that is essential for cell survival [53]. Next to this, membrane pore formation complicates the detection of fusion with a content mixing assay (Section 2.11). Fused GUVs of Lira et al. adopted elongated shapes with aspect ratios ranging from ~1.1-2.5, where it is assumed that higher aspect ratios represent more fusion. This relatively large spread in aspect ratios could be due to unequal incorporation of charged lipids in GUVs [52].

Although the presented works show successful vesicle fusion with a relatively simple approach, it is questionable if oppositely charged membranes can be used in an integrated synthetic cell. The incorporation of some membrane proteins through charge-based fusion has been shown [e.g. in 43, 54], but these methods are less compatible with charged proteins. Another disadvantage of charge-mediated fusion is that it is limited by the magnitude of charge: fusion is likely to proceed until the fusion product is neutralised [52]. Since real cells contain ~20% negatively charged lipids [55], it is likely that an integrated synthetic cell requires a similar fraction of charged lipids. Neutralisation of such a synthetic cell upon charge-mediated fusion might diminish its living-like properties, and would prevent the possibility to introduce an additional cycle of fusion.

2.9 DNA-mediated membrane fusion

In living cells, SNARE proteins are able to pull two lipid bi-layers into proximity of each other (Figure 2.6a) [23]. As binding of SNARE proteins to opposite membranes generates tension, they facilitate membrane fusion [56]. Researchers showed successful *in vitro* vesicle fusion mediated by SNARE proteins with a timescale of minutes [57, 58], where the fusion process was accelerated by Xu et al. through the design of a DNA-lipid tether that supports SNARE proteins [56]. Their approach, however, still requires the purification of SNARE proteins, which is a time-intensive and expensive process [23].

DNA nanostructures are increasingly used in cellular reconstitution experiments to mimic proteins, since they can be readily synthesised with desired modifications and require no complicated purification processes [23]. To reconstitute membrane fusion, Stengel et al. mimicked the SNARE protein complex by using two complementary membrane-anchored single-stranded DNAs (ssDNAs) [59]. Through DNA hybridisation, the vesicles are brought into proximity, allowing for fusion (Figure 2.6b). Using two unique complementary strands allows for a selective fusion mechanism, where one sequence is incorporated in GUVs, while the other can be incorporated in LUVs. Furthermore, this approach allows for many modifications in terms of anchor selection and orientation, sequence code and length, DNA coverage on the membrane, lipid composition and environmental conditions. By optimising these factors, fusion efficiency can be enhanced. In this section, we will discuss these factors together with the issue of vesicle leakage observed in DNA-mediated fusion experiments.

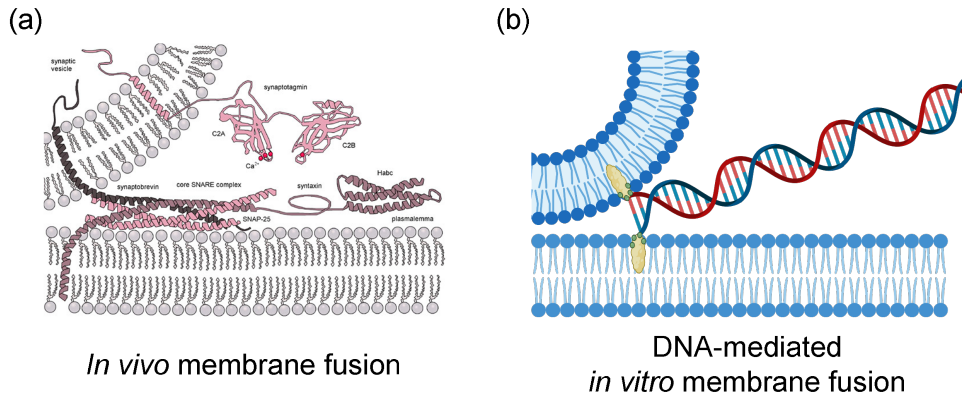


Figure 2.6 *In vitro* membrane fusion mimics the *in vivo* process. (a) *In vivo* membrane fusion is facilitated by SNARE proteins anchoring fusing membranes. Figure retrieved from Lychevski [60]. (b) *In vitro* membrane fusion can be achieved by mimicking SNARE proteins using DNA anchors. In this schematic, two ssDNAs are anchored with a single cholesterol tag. Figure made with BioRender.

2.9.1 Type of membrane anchors

DNA-mediated vesicle fusion requires the ssDNA to be incorporated in the membrane. There are various methods to anchor the DNA. Frequently used anchors are a single or dual cholesterol tag [e.g. 16, 59], where the hydrophobic part of this sterol spontaneously inserts in the lipid bi-layer. To incorporate the bivalent cholesterol anchor in the membrane, it has to be attached to a piece of double-stranded DNA (dsDNA), that continues into a piece of ssDNA required for vesicle docking (Figure 2.7a). Here, vesicle docking is the state where vesicles are bound to each other, but where the membranes are not merged in any way. Stengel et al. showed that for 100 nm LUVs with a bivalent cholesterol anchor, the level of content mixing after ~1 hr increased from ~7 to ~17% compared to a single cholesterol anchor [61]. The authors hypothesise that DNA hybridisation puts strain on the attached membranes. While using a single cholesterol anchor, this strain could be released by one of the cholesterol anchors leaving the membrane, thereby releasing the vesicle to which it was attached (Figure 2.7b). Since the released cholesterol anchor still prefers a hydrophobic environment, it is likely that it will shuttle to the other membrane. Stengel et al. proposed that a bivalent cholesterol anchor prevents this shuttling, either through an increased strain resistance, or through a lowered fusion timescale [61]. In a paper where liquid-ordered GUVs were fused with liquid-disordered 100 nm LUVs, however, incorporation of a single cholesterol or tocopherol-tagged ssDNA was found sufficient to achieve complete and reproducible fusion [16].

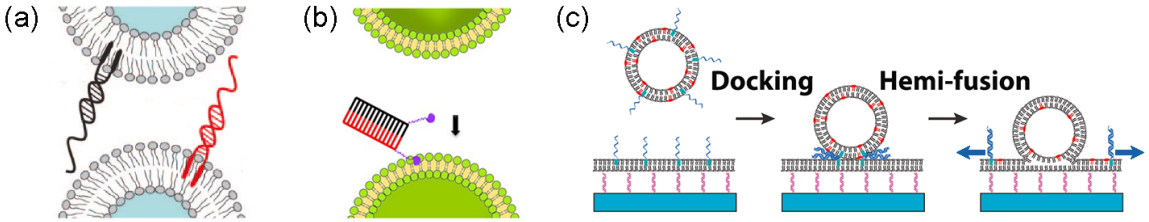


Figure 2.7 (a) Design of a bivalent cholesterol membrane anchor, that starts with a piece of dsDNA and continues into a piece of ssDNA required for hybridisation. (b) Shuttling from one membrane to the other membrane can occur for single cholesterol anchors to release membrane strain. (c) A short membrane anchor can result in diffusion of the hybridised DNA strand, thereby preventing fusion completion. Sub-figure (a) is adapted from Stengel et al. [61], (b) from Meng et al. [62], and (c) from Flavier and Boxer [63].

Instead of cholesterol, other amphipathic molecules can be used as membrane anchors. Flavier and Boxer showed that the length of the membrane anchor affects fusion efficiency [63]. In earlier work, they achieved fusion between 100 nm LUVs and a tethered lipid-bilayer by tagging ssDNA with diglycerol [64]. However, only 5% of binding events resulted in full fusion. Flavier and Boxer hypothesised that the short length of the diglyercol anchor, only spanning the outer leaflet of the lipid bi-layer, allowed for free diffusion of the anchor along the tethered bi-layer upon outer leaflet merging. Consequently,

hybridised DNA could move away from the fusing membranes, thereby preventing fusion completion (Figure 2.7c). To overcome this problem, they tagged ssDNA with a solanesol anchor, which is sufficiently long to span the entire lipid bi-layer. With a comparable experimental set-up, the fusion efficiency increased by ~2-3 fold. One side-note here is that membranes formed by EggPC were used in the diglyercol experiment, whereas the solanesol experiment used a membrane composition of DOPC/DOPE/cholesterol in a 2:1:1 ratio. Possibly, the presence of DOPE lipids contributed to the increased fusion efficiency (Section 2.6) [40]. Meng et al. designed an even more robust membrane anchor [62]. They tagged the four last nucleotides of their ssDNA with the hydrophobic molecule dodec-1-yne, thereby creating a quadruple-membrane anchor. With fluorescence resonance energy transfer (FRET) experiments, it was determined that ssDNA tagged with the quadruple anchor remained incorporated in the membrane for at least 24 hours. Content mixing of 100 nm LUVs increased from 8 to 29% for this quadruple anchor compared to a bivalent dodec-1-yne anchor.

2.9.2 Type of ssDNA sequence and anchor orientation

Other interesting insights for DNA-mediated vesicle fusion, and for membrane fusion in general, were provided by Chan et al. [65]. By comparing complementary non-repeating DNA sequences, i.e. sequences built up from all four bases, with complementary sequences consisting purely out of adenine (A) or thymine (T) bases, they found that the latter results in a dramatic increase of lipid mixing (~87% vs. ~48%) and content mixing (~15% vs. ~3%) (LUV-LUV fusion, d = ~120 nm). The authors hypothesise that fusion is enhanced for the poly A/T sequences because they do not have to perfectly overlap to allow binding, thereby releasing geometrical constraints for docking and thus fusion. Based on these results, they investigated if fusion is also enhanced for DNA strands with a partial non-complementary sequence [66]. Here, they performed SUV-SUV fusion experiments (d = 70-100 nm) with varying lengths of a non-complementary linker (Figure 2.8a). Vesicle docking increased with linker length, probably because of the flexibility of the non-complementary sequence. Lipid and content mixing, however, decreased with linker length (Figure 2.8b), stressing the importance of membrane proximity over strand flexibility for vesicle fusion.

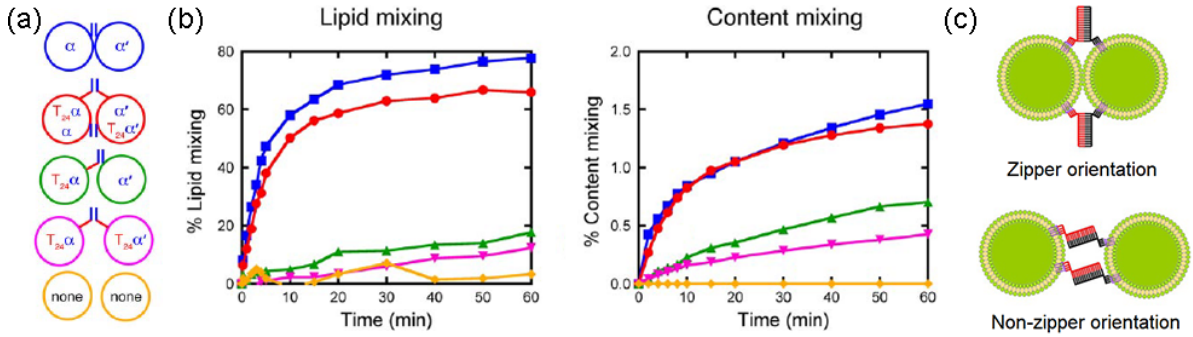


Figure 2.8 (a) Addition of non-complementary linkers (in red) to complementary DNA (in blue) to study the effects of strand flexibility and membrane proximity on fusion. (b) Measured lipid and content mixing rates corresponding to sub-figure (a). (c) The zipper orientation brings membrane closer to each other compared to the non-zipper orientation. Sub-figures (a) and (b) are retrieved from Chan et al. [66], sub-figure (c) adapted from Meng et al. [62].

The insight that membrane proximity is pivotal for efficient fusion was subsequently utilised in the design of ssDNAs: by attaching membrane anchors to the ssDNA sequence in a specific orientation, the distance between two membranes upon DNA hybridisation can be decreased. Here, we distinguish the zipper orientation, where the anchor is attached to the 5' end of one ssDNA and the 3' end of the other ssDNA, and the non-zipper orientation, where the anchors are attached to the 5' end of both ssDNAs. Since DNA hybridises in anti-parallel direction, it binds in a zipper-like fashion for the zipper orientation, pulling the membranes closer to each other compared to the non-zipper orientation (Figure 2.8c). Compared to the non-zipper orientation, the zipper orientation showed increased content mixing for LUV-LUV fusion (d = 120-130 nm [62, 65]) and fusion between LUVs (d = 100 nm) and a supported lipid bi-layer (SLB, Figure 2.9a). Furthermore, the zipper conformation was successfully utilised in the fusion of liquid-ordered GUVs with liquid-disordered 100 nm LUVs [16].

2.9.3 DNA density

Other papers investigated how fusion is influenced by DNA density on the membrane. In these papers, DNA coverage of the vesicle membrane was estimated from the molar ratios of lipids and DNA used. Here, it was generally assumed that all DNA was incorporated, because they used irreversible membrane anchors. Stengel et al. studied the effect of DNA density on LUV-LUV fusion (d = 100 nm) [61]. They found that ~13 DNA strands per vesicle were sufficient to gain a lipid mixing efficiency close to its maximum value, where ~16-17% of vesicles showed lipid mixing; comparable levels of lipid mixing were observed for ~100 DNA strands with a similar timescale. For LUVs of a comparable size (d = ~120 nm), however, Chan et al. found that 100 DNA strands per vesicle were required to obtain maximum levels of lipid mixing [65]. By using 10, 50, or 100 DNA strands per vesicle, they found 32%, 43%, and 48% of LUVs exhibiting lipid mixing when using non-repeating sequences, respectively. When using poly A/T sequences, they found values of 67%, 80%, and 87% for lipid mixing, respectively.

Another difference between the studies of Stengel et al. and Chan et al. was found in terms of fusion efficiency: Stengel et al. found that usage of 12 DNA strands per vesicle was sufficient to obtain the maximum level of content mixing, whereas fusion efficiency increased for up to 100 DNA strands per vesicle in the case of Chan et al. A difference in experimental set-up between the two studies is that Stengel et al. used a bivalent cholesterol membrane anchor, whereas Chan et al. used a longer anchor called lipid phosphoramidite. Possibly, lipid and content mixing was limited by the shorter bivalent cholesterol anchor for Stengel et al., thereby observing less dependence on DNA density.

In another article, Simonsson et al. investigated the effect of DNA density on SLB-LUV fusion (d_{LUV} = 100 nm) by varying the level of DNA coverage on the LUV membrane [67]. In this experiment, vesicle docking was promoted by addition of Ca^{2+} ions, a fusion strategy that was previously utilised in combination with anionic lipids instead of DNA molecules [68]. Contrary to the studies we discussed previously, Simonsson et al. found a lower and upper limit of DNA density required for successful fusion. They specifically found that 10-16 DNA strands were required to obtain full fusion, where fusion was detected for 17% of the vesicles. For lower and higher DNA densities, no fusion was detected. The lower limit of DNA density is explained by two reasons: a minimal amount of DNA strands is required to successfully tether the LUV to the SLB and to provide sufficient lateral membrane stress required for fusion. It was hypothesised that the upper limit is caused by steric hindrance and electric repulsion of DNA, thereby preventing the LUV membrane to get sufficiently close to the SLB for fusion. The authors postulate that no upper limit was observed in the LUV-LUV fusion experiment by Stengel et al., since each LUV was able to fuse with multiple LUVs in that experiment, thereby increasing the amount of utilised DNA [67]. However, one would still expect similar effects of steric hindrance and electric repulsion for LUV-LUV fusion if these are the genuine factors causing the upper limit for SLB-LUV fusion, albeit at a higher DNA density.

Lengerich et al. studied the effect of DNA density on the fusion of SUVs (d = ~ 50 nm) with a tethered lipid bi-layer membrane (tBLM, Figure 2.9b) [69]. Contrary to SLBs, tBLMs are covalently tethered to a support, thereby creating a more stable structure and a sub-membrane space [70]. Similar to the studies we discussed previously, Lengerich et al. estimated the amount of DNA strands per vesicle through the lipid and DNA molar ratios. The estimations were confirmed by using a fluorescent DNA-lipid, but showed a broad distribution (see Figure 2.9c for a representative example). Contrary to the other studies discussed, Lengerich et al. found little effect of DNA density on lipid mixing: similar levels of lipid mixing were obtained over a range of 1 to 65 DNA strands per vesicle. Full fusion was observed for only ~5% of the vesicles, and this event neither depended on DNA density.

To explain the lack of a dependence on DNA density for tBLM-SUV fusion, it makes most sense to compare the results with the SLB-LUV study by Simonsson et al. Possibly, the smaller vesicle size of the tBLM-SUV experiment (~ 50 nm vs. 100 nm) and the shorter DNA sequence (24 vs. 39 base pairs) resulted in less steric hindrance and electric repulsion, thereby eliminating the upper limit for DNA density that was observed in the SLB-LUV experiment. Similarly, a smaller vesicle size might have excluded a lower limit for DNA density, since small vesicles were found to be more fusogenic (Section 2.6).

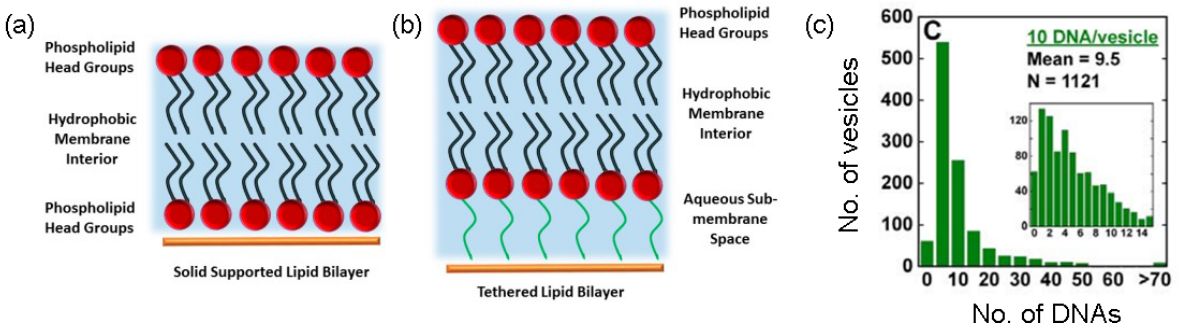


Figure 2.9 (a) A supported lipid bi-layer (SLB) floats on a solid support. (b) A tethered lipid bi-layer membrane (tBLM) is covalently attached to a support, thereby creating a more stable structure and an aqueous sub-membrane space. (c) Number of DNAs measured for vesicles where 10 DNAs/vesicle were expected. The mean number of DNAs/vesicle is 10, but the relationship shows a broad distribution. Sub-figures (a) and (b) retrieved from Andersson and Köper [70], sub-figure (c) retrieved from Lengerich et al. [69].

2.9.4 Lipid composition and environmental conditions

We already discussed the effect of lipid composition on membrane fusion in Section 2.6, where we described that there is a general consensus on the requirement of negative curvature for hemifusion. In line with this, Stengel et al. found increased levels of DNA-mediated lipid mixing for membranes containing more DOPE and cholesterol [59], two lipids with negative spontaneous curvature [71]. Specifically, they found that lipid mixing increased from ~8 to ~28% for 100 nm LUVs consisting out of DOPC/DOPE/cholesterol in a 2:1:1 molar ratio compared to LUVs built up from DOPC/DOPE or DOPC/cholesterol in a 3:1 molar ratio.

Next to the effect of lipid composition on membrane fusion, lipid composition influences DNA incorporation. Stengel et al. found that DNA with a bivalent cholesterol anchor is more readily incorporated in membranes containing cholesterol [59]. From FRET experiments, however, the authors concluded that all DNA is incorporated over time for the different membrane compositions tested. Based on this, they state that the differences observed in lipid mixing are caused by physical characteristics associated with the membrane compositions. They specifically hypothesise that enhanced lipid mixing for membranes containing cone-shaped lipids is caused by the formation of phase-separated lipid rafts [59]. Related to this, Dreher et al. showed the successful fusion of liquid-ordered GUVs with liquid-disordered 100 nm LUVs, thereby forming a phase-separated fusion product [16]. The authors hypothesise that upon the first GUV-LUV fusion event, the energy barrier for consecutive fusion events is lowered by the generation of a line tension on the phase boundary. An enhanced fusion efficiency between phase-separated GUVs and 100 nm LUVs was also found by Peruzzi et al. [72]. Contrary to Dreher et al., however, they only found enhanced fusion when both of the fusing vesicles contained liquid-ordered and liquid-disordered phases. They hypothesise that fusion is enhanced for phase-separated vesicles, since the fusion of liquid-ordered domains with liquid-disordered domains decreases the total length of phase boundaries, thereby decreasing the associated free energy.

The influence of an external environmental factor on DNA-mediated membrane fusion was studied by Morzy et al. [73]. In their paper, they studied the requirement of cations for the incorporation of cholesterol-tagged DNA in membranes. Here, they found that ~4 mM of divalent cations is required to successfully incorporate DNA, whereas for monovalent cations, a concentration of ~100 mM is required to maximise DNA incorporation. The observed effect is explained by electrostatic repulsion between negatively charged DNA and vesicles with a negative surface charge. To successfully incorporate DNA, screening of the DNA by cations is required. Note that Morzy et al. measured negative surface charges for vesicles containing anionic lipids, but also for vesicles composed purely of zwitterionic DPPC lipids. They used a pH of 7.5 throughout their experiments, which is in the acidic range of living systems [74], thereby making their findings relevant for a synthetic cell.

2.9.5 Vesicle leakage

DNA incorporation can cause increased permeability, potentially caused by transient pore formation [62]. This is not only troublesome for the delivery of cargo through vesicle fusion, but also for the detection of fusion using a content mixing assay. Chan et al. for example reported that 10% of contents used to detect fusion leaked from the vesicles after one hour [66]. Stengel et al. compared content mixing rates with leakage rates [61]. Here, they found that content mixing generally precedes vesicle leakage, but that especially in the case of using a single cholesterol anchor, the final leakage rate is higher than the final content mixing rate. Meng et al. minimised vesicle leakage to less than 2% by utilising the quadruple membrane anchor described in Subsection 2.9.1. They hypothesise that a more robust anchor is less likely to leave the membrane, thereby decreasing the chance of pore formation.

2.10 Epifluorescence microscopy

Since GUVs are micron-sized, they conveniently allow for visualisation by optical microscopy. We used epifluorescence microscopy in this project to visualise vesicles and to detect DNA incorporation, GUV-LUV binding and GUV-LUV fusion. Epifluorescence microscopy is based on the light emission of fluorescent molecules upon illuminating them with light of a specific shorter wavelength [75]. The light emitted by fluorophores can be captured with a camera to construct an image. The light with which the fluorescent molecules are excited and the light they emit traverse through the same objective lens [76]. Since the excitation light has a significantly higher intensity than the emission light, spectral emission filters are used to filter out the excitation light, thereby only capturing the signal from the fluorescent molecules. The maximum resolution is constrained by Abbe's limit, which is in the order of 150-300 nm for visible light [77].

Excitation and emission spectra can be broad and depend on the dye of choice [75]. A common issue with epifluorescence microscopy is fluorescence crosstalk. This phenomenon is caused by overlapping excitation and emission spectra of fluorescent molecules, complicating the optical separation of the two dyes [78]. Since this study involves quantitative fluorescence imaging, we had to be absolutely sure that cross-talk between dyes was minimal. This was ensured by selecting lipid dyes that showed little to no fluorescence crosstalk.

2.11 Fusion detection methods

Scientists typically use two different methods to detect membrane fusion: lipid mixing and content mixing. These methods have mainly been used to detect fusion between two similarly-sized vesicles [79]. The first of these methods is a lipid mixing assay, where fusing membranes are both endowed with a different lipid dye [48]. This method is especially useful to distinguish between vesicle docking and hemifusion. In the lipid mixing assay, two vesicles are in the docking state when they only contain their own lipid dye (Figure 2.10a), and in the hemifusion state when their outer leaflets contain a mix of the two lipid dyes (Figure 2.10b). Full fusion, where the two membrane leaflets have fully merged, can subsequently be detected if both leaflets contain a mix of the two lipid dyes (Figure 2.10c). In this case, lipid mixing between the outer and inner leaflets of two fusing membranes has taken place.

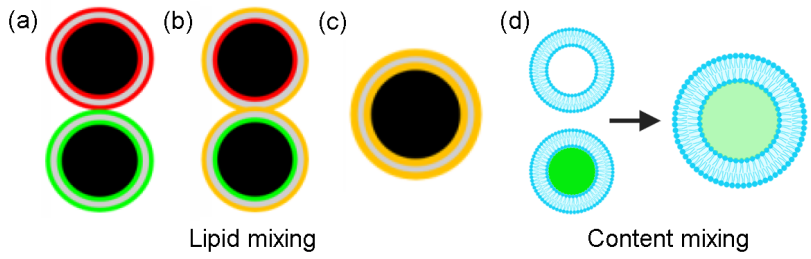


Figure 2.10 Lipid and content mixing assays to detect vesicle fusion. Lipid mixing is able to distinguish between (a) vesicle docking, (b) hemifusion, and (c) full fusion. Figure adapted from Arribas Perez and Beales [48]. (d) Content mixing of vesicles results in a diluted fluorescent signal in the fusion product (when using a non-self-quenching dye). Figure made with BioRender.

The content mixing assay detects full fusion only. A fluorescent dye is encapsulated in one vesicle type, and is only transferred to the other vesicle upon full fusion [80]. To detect fusion, the signal of the fluorescent dye decreases upon fusion by dilution (Figure 2.10d), or increases when using a self-quenching dye [81]. In this project, we chose to adopt a content mixing assay, because we were specifically interested in the final state of fusion, where both lipid leaflets have mixed. In this content mixing assay, we encapsulated a fluorescent dye in our LUVs. We could subsequently detect fusion through the observation of this dye in the interior of our GUVs. We were thus not able to distinguish between vesicle docking and hemifusion.

2.12 Coupling tension and DNA-mediated fusion to acquire membrane growth

In this chapter, we discussed multiple strategies to reconstitute membrane fusion. Although fusion approaches based on charge and DNA have been described extensively in literature, the majority of these works involved LUV-LUV or SUV-SUV fusion. Little work has been devoted to GUV-LUV/SUV fusion and the amount of membrane growth that can be achieved with this process. Recent studies that did investigate GUV-LUV/SUV fusion used mechanisms based on complementary synthetic peptides [82], or on a combination of amphipilic nanoparticles and calcium [83]. However, the fraction of fused vesicles was not quantified in these studies. Next to this, the studies mainly focused on utilising GUV-LUV/SUV as a tool for drug delivery, where a large number of fusion events might be less relevant than for applications of membrane growth. In another recent article, Tivony et al. showcased charge-mediated GUV-SUV fusion on a chip [84]. However, for the arguments given in Section 2.8, we believe that this fusion strategy is less compatible with an integrated synthetic cell. In Section 2.7, we discussed the fusion of GUVs with SUVs with a tension-mediated approach [30]. Although this method shows considerable membrane growth for part of the vesicle population, it lacks a selective fusion mechanism, and it has a relatively long fusion timescale.

To overcome the presented issues, we combined the tension-based fusion strategy with a DNA-mediated approach in this study. By using complementary DNA strands, we utilised a selective fusion mechanism that, based on the fusion timescales observed for DNA-mediated fusion (typically within one hour, e.g. in [59, 61, 62, 65, 66]), had the potential to accelerate the process of membrane growth. Furthermore, many parameters could be tweaked for the DNA-mediated approach, offering opportunities to optimise fusion efficiency.

In Section 2.9, we discussed the article by Dreher et al., where they fused liquid-ordered GUVs with liquid-disordered LUVs [16]. In this work, it is mentioned that full fusion was achieved reproducibly, but fractions of fused vesicles in a population were not quantified. Based on the two-dimensional projection of a single GUV, the number of GUV-LUV fusion events for a single GUV was estimated, but the general amount of membrane growth obtained is not provided. Next to this, the influence of DNA density, tension, and LUV size on DNA-mediated GUV-LUV fusion remains unknown. We therefore studied the effects of these and other parameters in this study, through which we explored if our fusion approach is compatible with other synthetic cell modules. Moreover, we quantified fusion efficiency, and we made a general estimate on the amount of membrane growth we could obtain with our novel fusion protocol.

The main research question of this project as well as the research aims are summarised in Figure 2.11. For the sake of simplicity, we studied vesicles fully built up from DOPC lipids and with a single cholesterol anchor for the largest part of this study. Using this experimental set-up, we already found a considerable fusion rate with up to ~30% of GUVs fused under optimal conditions within a time frame of less than two hours. In the future, the developed fusion protocol can be further optimised to gain an even higher fusion efficiency.

How to obtain *in vitro* membrane growth required for a synthetic cell to divide?

1. Explore the parameter spaces of DNA and tension-mediated fusion
→ Boost fusion efficiency
→ Provide a selective fusion mechanism
→ Accelerate the process of membrane growth
→ Offers many possibilities for modifications

2. Study the comptability of the two fusion strategies with each other and with an integrated synthetic cell

3. Quantify fusion efficiency

4. Make an estimate on the amount of membrane growth

Figure 2.11 Main research question and research aims of this project.

3
Materials and methods

3.1 Lipids and chemicals

In Tables 3.1 and 3.2, an overview of the (fluorescent) lipids, proteins and chemicals used in the project is provided. Abbreviations used in this chapter that are not displayed in these tables, can be found in the "List of abbreviations and glossary" (p. xi). Lipids were diluted in chloroform and stored in glass vials at -20 ℃ under argon.

Compound	Abbreviation	Molecular weight (g/mol)	Supplier
1,2-dioleoyl-sn-glycero -3-phosphoethanolamine-N-(biotinyl) (sodium salt)	Biotinyl PE	992	Avanti Polar Lipis, Inc.
Cholesterol	N.A.	387	Sigma-Aldrich
1,2-dioleoyl-sn-glycero-3-phosphocholine	DOPC	786	Avanti Polar Lipids, Inc.
1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt)	DOPS	810	Avanti Polar Lipids, Inc.
1,2-dioleoyl-3-trimethyl-ammonium-propane (chloride salt)	DOTAP	698	Avanti Polar Lipids, Inc.
1-palmitoyl-2-oleoyl-sn-glycero -3-phosphoethanolamine	POPE	718	Avanti Polar Lipids, Inc.
Beta-casein	N.A.	$24 \cdot 10^3$	Sigma-Aldrich
Cobalt(II) chloride anhydrous	$CoCl_2$	130	Sigma-Aldrich
p-Xylene-bis(N-pyridinium bromide)	DPX	422	Sigma-Aldrich
Ethylenediaminetetraacetic acid, 0.5 M sterile solution, pH 8.0	EDTA	292	AMRESCO
D-(+)-Glucose	Glucose	180	Fluka® Sigma-Aldrich
Potassium chloride	KCl	75	Sigma-Aldrich
Recombinant Proteinase K Solution (20 mg/mL)	Proteinase K	29	Thermo Fisher Scientific
Polivinyl alcohol 98% hydrolised	PVA	$145 \cdot 10^3$	VW International BV
Sucrose	N.A.	342	Sigma-Aldrich
Tris hydrochloride	Tris	158	Sigma-Aldrich

Table 3.1 Table of non-fluorescent compounds used in the project.

Compound	Abbreviation	Excitation wavelength (nm)	Emission wavelength (nm)	Molecular weight (g/mol)	Supplier
DOPE-Atto488	Atto488	500	520	1316	ATTO-TEC GmbH
DOPE-Atto655	Atto655	663	680	1368	ATTO-TEC GmbH
1,2-dioleoyl-sn-glycero-3-phospho-ethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)	RhoPE	560	583	1301	Avanti Polar Lipids, Inc.
Fluorescein-bis-(methyliminodi-acetic acid)	Calcein	470	509	623	Sigma-Aldrich
Aequorea victoria GFP Protein (His Tag), Lyophilized	His-GFP	395	508	$29 \cdot 10^3$	Thermo Fisher Scientific
8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt	HPTS	454	511	524	Sigma-Aldrich
Octadecyl Rhodamine B Chloride	R18	556	578	732	Biotium
Streptavidin, Alexa Fluor™ 488 conjugate	Streptavidin	499	520	$60 \cdot 10^3$	Thermo Fisher Scientific
Sulforhodamine B monosodium salt	Sulforhodamine B	565	586	581	Sigma-Aldrich

Table 3.2 Table of fluorescent compounds used in the project.

3.2 GUV preparation

3.2.1 Gel-assisted swelling

Unless stated differently, GUVs were prepared with the polyvinyl alcohol (PVA) gel-assisted swelling method [26]. This vesicle formation method was used since it is a relatively simple and quick technique that is robust to changing lipid compositions and swelling solution contents [85]. PVA gel was prepared by stirring 5% (w/v) PVA in a 200 mM sucrose solution in water at 90 ℃ until it was fully dissolved. The PVA stock solution was stored in the fridge. Since the PVA gel has a relatively high viscosity at this temperature, a 1.5 mL Eppendorf Tube ® with PVA gel was typically stored at room temperature to allow for easier pipetting. This also enhanced spreading of the liquid gel upon application.

Cover glasses (22x22 mm, No. 1.5H, Paul Marienfeld GmbH & Co. KG) were first cleaned with ethanol and Milli-Q water and dried under a stream of nitrogen gas. To improve the wettability of the cover glasses, they were plasma cleaned for 30 seconds at a radio frequency (RF) of 20 (Plasma Prep III, SPI Supplies). Then, 100 µL PVA solution was spread over each coverslip. The gel was solidified by placing the cover glasses in the oven for 30 minutes at 50 ℃. Next, 10 µL of the desired lipid stock solution (1 mg/mL lipids) was spread on top of the dried PVA gel using a glass pipette until the solution was visually evaporated. To promote further evaporation of the organic solvent, the cover glasses were put under vacuum in the desiccator for 30 minutes. The dried lipids were then incubated for one hour with 300 µL GUV swelling buffer per sample, which was placed on top of the gel (Table 3.3). Buffer was applied gently to not disturb the dried lipid film. Here, the individual GUV samples were kept in different chambers of a compartmentalised petri dish to avoid exchange of lipids between different samples. During buffer incubation, the lid of the petri dish was closed to prevent evaporation. After incubation, the GUVs were retrieved by manoeuvring the vesicle solution to the corner of the petri dish chamber. The vesicle solution was then pipetted once over the coverslip to promote GUV detachment from the gel. The vesicles could then be harvested from the corner of the chamber. GUVs were stored in the fridge and used within one day.

Buffer name	Buffer composition	Theoretical osmolarity (mOsm)
GUV swelling buffer	100 mM KCl + 100 mM sucrose + 10 mM Tris (pH 7.4)	320
Observation buffer (OBS)	100 mM KCl + 100 mM glucose + 10 mM Tris (pH 7.4)	320
Quenching observation buffer (Quenching OBS)	100 mM KCl + 100 mM glucose + 10 mM Tris (pH 7.4) + 5 mM DPX (DNA-based experiments) or 10.7 mM DPX (charge-based experiments)	325 (DNA-based experiments) or 330.7 (charge-based experiments)
SUV/LUV buffer	100 mM KCl + 10 mM Tris (pH 7.4)	220
Fluorescent SUV/LUV buffer	100 mM KCl + 10 mM Tris (pH 7.4) + 10 mM HPTS	260

Table 3.3 Composition of the buffers most frequently used in the project. Theoretical osmolarities were determined from the osmolarities of the individual buffer components. For experiments where the effect of osmotic conditions on DNA incorporation was investigated, osmolarities were experimentally verified (Tables 3.9 and 3.11).

3.2.2 GUV lipid compositions

An overview of the different GUV lipid compositions used in the project can be found in Table 3.4. In this thesis, the different types of GUVs will be described with their "GUV name". Depending on the specific experiment, different lipid compositions were used to either prevent fluorescence crosstalk, or to test the influence of lipid composition on fusion rate. All GUV stock solutions had a total lipid concentration of 1 mg/mL. Most stock solutions contain a lipid dye to allow for fluorescent imaging of the vesicles. Atto dyes (ATTO-TEC GmbH) were used for their high photostability. The imaging settings used to visualise each GUV type are displayed in Table 3.4 as well, and the details of each imaging setting can be found in Table 3.6.

GUV name	Lipid composition	Fluorescent label imaging settings
Unlabelled GUV	100% DOPC	N.A.
0.1% Atto488 GUV	99.9% DOPC + 0.1% Atto488	GUV-0.1%Atto488
0.5% Atto488 GUV	99.5% DOPC + 0.5% Atto488	GUV-0.5%Atto488
0.01% Atto655 GUV	99.99% DOPC + 0.01% Atto655	GUV-0.01%Atto655
0.1% Atto655 GUV	99.9% DOPC + 0.1% Atto655	GUV-0.1%Atto655
0.5% Atto655 GUV	99.5% DOPC + 0.5% Atto655	GUV-0.5%Atto655
PE-containing GUV	69.5% DOPC + 30% POPE + 0.5% Atto488	GUV-0.5%Atto488
Physiologically relevant GUV	59.5% DOPC + 20% DOPS + 20% Cholesterol + 0.5% Atto488	GUV-0.5%Atto488
R18 GUV	95% DOPC + 5% R18	GUV-R18
20% PS GUV	79.9% DOPC + 20% DOPS + 0.1% Atto655	GUV-0.1%Atto655
40% PS GUV	59.9% DOPC + 40% DOPS + 0.1% Atto655	GUV-0.1%Atto655

Table 3.4 Lipid compositions and fluorescent label imaging settings of GUVs used in the project. Percentages indicate mole percentages. The lipid composition of physiologically relevant GUVs was based on work from Symons et al. [55]. Details of fluorescent label imaging settings can be found in Table 3.6.

3.3 SUV and LUV preparation

3.3.1 Lipid drying

We used small vesicles of three different sizes in this project: SUVs of 30 nm and LUVs of 100 and 200 nm were used. To prepare SUVs and LUVs, 5 mL Pyrex ® glass tubes were first cleaned with tap water and soap. Then, minerals from the tap water were flushed away with demi water. Next, the glass tubes were cleaned with ethanol, acetone and Milli-Q water. After cleaning, the hemispherical parts of the glass tubes were filled with chloroform. The desired lipid solutions were then added. Typical quantities added were 29 µL DOPC at 25 mg/mL and 6.5-62 µL fluorescently-tagged lipid at 0.1 mg/mL, depending on the final fraction of lipid dye in the vesicles. The lipid compositions of the

different types of SUVs and LUVs can be found in Table 3.5, as well as the imaging settings to visualise the vesicles.

SUV/LUV name	Lipid composition	Fluorescent label imaging settings
Unlabelled LUV	100% DOPC	N.A.
RhoPE SUV/LUV	99.95% DOPC + 0.05% RhoPE	SUV/LUV-RhoPE
Atto655 LUV	99.95% DOPC + 0.05% Atto655	SUV/LUV-Atto655
Biotinyl PE LUV	79.95% DOPC + 20% Biotinyl PE + 0.05% Atto655	SUV/LUV-Atto655
20% TAP SUV/LUV	79.95% DOPC + 20% DOTAP + 0.05% RhoPE	SUV/LUV-RhoPE
40% TAP SUV	59.95% DOPC + 40% DOTAP + 0.05% RhoPE	SUV/LUV-RhoPE

Table 3.5 Lipid compositions and fluorescent label imaging settings of SUVs and LUVs used in the project. Percentages indicate mole percentages. Details of fluorescent label imaging settings can be found in Table 3.6. SUVs were extruded through a 30 nm pore, and LUVs through a 100 or 200 nm pore (Subsection 3.3.2).

After gently swirling the glass tubes to allow for proper dilution of the lipids in the chloroform, the lipids were dried under a stream of nitrogen. The initial addition of chloroform to the glass tubes was necessary to properly mix lipids and resulted in a larger surface area of dried lipids compared to only adding the lipid solutions. Further evaporation was promoted by placing the glass tubes in a desiccator. After two hours, 1.5 mL SUV/LUV buffer (Table 3.3) was added to the glass tubes to obtain a lipid concentration of 0.5 mg/mL. The buffer was incubated for two minutes. Next, the solution was vortexed for 30 seconds and put in a ultrasonic bath sonicator for 30 seconds (Branson 2510 Ultrasonic Cleaner, Marshall Scientific), resulting in multilamellar vesicles.

3.3.2 Vesicle extrusion

The multilamellar vesicles were extruded with the Avanti Mini Extruder to form SUVs or LUVs. To assemble the extruder, 10 mm filter supports (Avanti Polar Lipids, Inc.) were first pre-wetted in SUV/LUV buffer and then put in the middle of the black o-rings of the cylindrical white teflon parts. A polycarbonate membrane (Whatman plc.) with a pore size of the desired vesicle diameter was pre-wetted in the same buffer and put on top of one of the teflon parts. The two teflon parts were then mounted on top of each other, with the two o-rings being in contact to attain waterproofness. Subsequently, the teflon parts were put in the metal holder and placed in the heating block of the extruder. An empty syringe was then gently pushed in the pinhole of the right teflon part, and a syringe that typically contained ~300 µL SUV/LUV buffer was pushed in the left pinhole.

To further pre-wet the extrusion chamber and to test for water-tight closure, the buffer was pushed ~5 times through the extruder. As extrusion sometimes required high forces, especially for 30 nm SUVs, we tested water-tightness of the extruder using higher compressive forces, i.e. by firmly holding one syringe while pushing the other. When the extruder showed no leakage next to the small amount of liquid that remained in the chamber (~10 µL), we emptied the syringe.

To start the formation of unilamellar vesicles and increase size monodispersity, the left syringe was filled with 1 mL of the vesicle solution suspension, obtained after vortexing as described above. The solution was typically extruded 21 times through the membrane with a pore size of 200, 100 or 30 nm, where the vesicle diameter decreased with smaller pore size as verified with DLS measurements (Section 4.2 and Appendix A). It was important to extrude the vesicle solution an odd number of times, since the starting syringe was contaminated with the initial sample. For SUVs, the solution was first extruded 21 times through a 100 nm pore, and then 21 times through a 30 nm pore, unless stated differently. In some cases, a lower number of extrusion steps was performed if there was sample leakage from the chamber, but this had little effect on the final vesicle size (Appendix A). SUVs and LUVs were stored in the fridge and were generally used within one day, since it was found that vesicle size distribution changed over longer time periods (Section 4.2). The only exception to this is the local injection experiment with 30 nm SUVs (Subsection D.1.4), where SUVs of one week old were used.

3.4 SUV and LUV characterisation

Dynamic light scattering (DLS) measurements were performed to determine the size of SUVs and LUVs (Zetasizer Nano ZS, Malvern Panalytical). For each measurement, 70 µL of the sample was pipetted in a disposable cuvette (ZEN0040, Malvern Panalytical). We ensured that the small triangle on the cuvette faced towards the front of the instrument, since measurement performance is improved by letting the light enter through this polished surface. The size of the vesicles was determined with the following settings:

- Sample settings
  - Material
    - ◇ Refractive index = 1.350
    - ◇ Absorption = 0.010
  - Dispersant
    - ◇ Osmolarity = 150 mM KCl (most closely matched available option)
    - ◇ Viscosity = 0.8733 cP
    - ◇ Refractive index = 1.332
  - Temperature = 25.0 ℃
  - Equilibirum time = 30 s
- Measurement settings
  - 173 ° Backscatter (NIBS default)
  - 2 measurements with automatic duration

The instrument software converted the raw data to particle size distributions (PSDs) based on measured intensity and the number of vesicles. The input for this analysis was the correlation function, which indicates the duration of a particle remaining in the same place in the sample. By fitting a polynomial to the log of this correlation function, where the polynomial constants were determined by the measurement settings and some instrument constants, the PSDs could be determined.

3.5 Microscopy set-up and settings

3.5.1 Chamber preparation

In the majority of the experiments, microscopy chambers with volumes of 20 and 200 µL were used. The only exception to this are the chambers used for micropipette experiments (Appendix D). The 200 µL chambers were used most frequently since they allowed for the addition of extra sample during experiments. The 20 µL chambers were prepared by first cleaning a cover glass (22x22 mm, No. 1.5H, Paul Marienfeld GmbH & Co. KG) with ethanol and Milli-Q water. Then, the cover glass was dried under a stream of nitrogen gas. A silicone spacer (Grace Bio-Labs reusable CultureWell™ gaskets, Sigma-Aldrich) was rinsed with isopropanol and dried using nitrogen gas as well. The spacer was then fixed on the cover glass by spontaneous adhesion, creating 20 µL microscopy chambers. The spacer chambers were passivated for at least 15 minutes with 20 µL 1 mg/mL beta-casein in an aqueous solution of 10 mM Tris (pH 7.4). Then, the chambers were flushed at least two times with 20 µL OBS, where liquids were removed using the tip of a tissue. When the samples were added, the spacer was covered with an additional cleaned cover glass to prevent evaporation.

200 µL microscopy chambers were created by cutting a 24x50 mm cover glass (No. 1.5H, Paul Marienfeld GmbH & Co. KG) to a 24x40 mm cover glass. This was done using an engraving pen (Sigma-Aldrich), and the cutting was performed on a on a Harris cutting mat (Sigma-Aldrich). Then, the glass slide was cleaned with ethanol and Milli-Q water, and dried under a stream of nitrogen gas. Next, the glass slide was placed in a custom-made metal holder. The chambers were prepared by cutting the bottom tip and lid of 0.2 mL thin-walled tubes with flat caps (Thermo Fisher Scientific). A thin layer

of two-component adhesive was then put on the largest opening of the cut tubes, through which they were fixed on the glass slide. Similar to the 20 µL chambers, the 200 µL chambers were passivated for at least 15 minutes with 1 mg/mL beta-casein in a solution of 10 mM Tris (pH 7.4), and flushed two or more times with OBS. The amount of beta-casein and OBS added varied per experiment and was equal to the total final sample composition, consisting of OBS and vesicle solutions.

3.5.2 Microscope

The "Minicell" microscopy set-up was used for all experiments that involved imaging (Nikon Eclipse Ti-E inverted microscope, ORCA-Flash4.0 LT+ Digital CMOS camera C11440m, Lumencor Spectra X light engine for LED monochromatic imaging). The microscope was used for brightfield and epifluorescence imaging with a 60x magnification water objective (CFI Plan Apochromat VS 60x WI, NA 1.00, Nikon) for micropipette aspiration experiments (Subsection D.1.3), and a 100x magnification oil objective for all other experiments (CFI Plan Apochromat VC 100x oil, NA 1.40, Nikon).

3.5.3 Fluorescent label imaging settings

The samples were observed and imaged using different fluorescent label imaging settings (Table 3.6), where the LED wavelengths used were selected based on the fluorescent compounds observed (Table 3.2). Here, we ensured to have sufficient signal but to prevent oversaturation. LED intensities and exposure times can deviate between experiments, if they had to be adapted to make the object under study properly visible to allow for visual inspection. In general, this was only the case for imaging channels where we were not interested in the absolute signal: either for the detection of GUVs for analysis (Section 3.12), or for the detection of HPTS, which was only used as a relative measure (Section 3.10). The adaption of these imaging settings did thus not affect the result in any way. When differences in LED intensities and exposure times did have an effect on experimental outcome, for example when comparing the level of GUV-LUV binding, we only compared images taken with identical settings.

Fluorescent label	LED wavelength (nm)	LED intensity (%)	LED exposure time (ms)	Filter used (colour)
Calcein	470 (charge) or 508 (DNA)	11 (charge) or 10 (DNA)	500 (charge) or 100 (DNA)	No (charge) or Yes (DNA, orange)
DNA-Atto488	508	10	100	Yes (orange)
GUV-0.1%Atto488	508	80 (Exceptions: 20 (samples 1 & 3) or 60 (samples 2 & 4) of Subsection 3.11.7)	100	Yes (orange)
GUV-0.5%Atto488	508	20	100	Yes (orange)
GUV-0.01%Atto655	640	5	100 (Exception: 10 (Subsection C.1.3))	No
GUV-0.1%Atto655	640	5-20 (indicated in main text)	100 (Exception: 500 (Subsection C.1.1, charge))	No
GUV-0.5%Atto655	640	5	10	No
GUV-R18	555	3	100	No
HPTS	440	1 (Exceptions: 2 (Subsections 3.11.4 & 3.11.7, Appendix B) or 10 (Subsection A.1.2))	10-100 (indicated in main text & Table 3.12	Yes (green)
Streptavidin-Alexa488	470	30	100	No
SUV/LUV-Atto655	640	40 (Exception: 10 (sample 3) of Subsection 3.11.7)	100	No
SUV/LUV-RhoPE	555	10	100 (Exception: 500 (Subsection C.1.1, charge)	No

Table 3.6 Fluorescent label imaging settings used in the project.

3.6 Single-stranded DNAs

An overview of the different ssDNAs used in the project is visible in Table 3.7 (biomers.net GmbH, HPLC purified). Both ssDNAs were conjugated with a cholesterol moiety to ensure membrane anchoring. By placing the cholesterol anchor for one strand on the 3' end, and for the other on the 5' end, anchors were oriented in the zipper orientation (Subsection 2.9.2). The ssDNAs we used for DNA-based experiments were adopted from Dreher et al. [16], who based their strand design on earlier work showing successful fusion of SUVs [66]. At the end of the project, we found out that the chol-DNA2-x strand we adopted from Dreher et al. contained an error (Subsection 5.8.2), but we still obtained considerable fusion rates (Section 4.7).

ssDNA name	DNA sequence	Number of bases
x-DNA1-chol	5'-TGGACATCAGAAAGGCACGACGA-Cholesterol-TEG-3'	23
chol-DNA2-x	5'-Cholesterol-TEG-TCCGTCGTGCCTTATTTCTGATGTCCA-3'	27
x-DNA1-Atto488	5'-TGGACATCAGAAAGGCACGACGA-Atto488-3'	23

Table 3.7 DNA sequences and number of bases of the ssDNAs used in the project. Strands were adapted from Dreher et al. [16], who based their strand design on Chan et al. [65]. The chol-DNA2-x strand contained an error (Subsection 2.9.2).

The ssDNAs were delivered as a powdery substance and were diluted to a concentration of 100 µM in 10 mM Tris (pH 7.4) after shortly spinning down the vials. The DNA solutions were stored in the fridge. In all experiments involving DNA, chol-DNA2-x was incorporated in the GUVs. For DNA incorporation experiments, x-DNA1-Atto488 was added to determine the level of DNA insertion into the GUV membrane. For vesicle binding and fusion experiments, x-DNA1-chol was added to SUVs or LUVs. Upon

mixing the GUVs with SUVs or LUVs, the SUVs or LUVs were binding to (and possibly fusing with) the GUVs through DNA hybridisation.

3.7 DNA incorporation

3.7.1 Overview and incubation time

To test the level of DNA incorporation in the GUV membrane, we added chol-DNA2-x and x-DNA1-Atto488 to GUV swelling buffer containing GUVs to a final concentration of 1 µM unless stated otherwise. The most frequently used DNA concentration of 1 µM could then for example be obtained by adding 1 µL of each ssDNA (100 µM stock concentration) to 98 µL GUVs. DNA was added to GUVs in a 1.5 mL Eppendorf Tube ® without mixing in presence or absence of OBS, and incubated overnight in the fridge, unless stated differently. An overview of the conditions for the different DNA incorporation experiments can be found in Table 3.8. After DNA incubation, the GUVs were added to 200 µL microscopy chambers. If no OBS was already added during DNA incubation, this was added to the chambers as well (see specific experiments for details).

The level of DNA incorporation could be determined by measuring the Atto488 fluorescent signal on the GUV membrane using the DNA-Atto488 imaging settings (Table 3.6). To confirm the absence of non-specific membrane localisation of x-DNA1-Atto488, we assessed that there was no Atto488 signal on the GUV membrane when no chol-DNA2-x was added. All chambers were closed with lids during measurements to prevent sample evaporation. To determine the incorporation time of DNA, images were taken at multiple delay times after mixing 1 µM DNA with 0.01% Atto655 GUVs.

Experiment	Type of GUVs	Sample composition during DNA incubation	DNA concentration (µM)	DNA incubation time
DNA incorporation time	0.01% Atto655 GUVs	78.4 µL GUVs in GUV swelling buffer + 0.8 µL of each ssDNA	1	5 min., 0.5 hr, 1 hr, 1.5 hr, or overnight
DNA concentration	Unlabelled GUVs	25 µL GUVs in GUV swelling buffer + DNA + OBS (total volume 100 µL)	0, 0.25, 0.5, 1, 2.5, or 5	Overnight
Osmotic conditions	0.01% Atto655 GUVs	10 µL GUVs in GUV swelling buffer + 0.4 µL of each ssDNA + 29.2 µL OBS (no DNA + 30 µL OBS for no DNA control)	1	Overnight

Table 3.8 Overview of the conditions used for the different DNA incorporation experiments. GUV compositions can be found in Table 3.4.

3.7.2 DNA concentration

The effect of DNA concentration on DNA incorporation was determined by adding 0, 0.25, 0.5, 1, 2.5, or 5 µM of chol-DNA2-x and x-DNA1-Atto488 to unlabelled GUVs in GUV swelling buffer and OBS, which are values around the 1 µM DNA used by Dreher et al. [16]. The amount of GUV solution was kept constant at 25 µL, since a difference in vesicle density could influence the level of DNA incorporation. The total volume of the samples was kept constant at 100 µL, where the amount of DNA stock solution to be added increased for higher DNA concentrations. Adding more DNA to GUVs for higher concentrations possibly had experimental side-effects, due to a decreased total osmolarity of the solution and increased dilution of GUV buffer components. In case of the highest DNA concentration used (5 µM), we estimate that the osmolarity was ~305 mOsm compared to ~320 mOsm when adding no DNA, and that GUV buffer components were diluted with a factor of ~0.05. To minimise experimental side-effects, the majority of DNA concentrations we tested were in the lower range regime (0-1 µM DNA).

3.7.3 Osmotic conditions

To investigate the effect of osmotic conditions on the level of DNA incorporation, 0.01% Atto655 GUVs in 10 µL GUV swelling buffer were, together with 0.4 µL of each ssDNA, added to 29.2 µL of observation buffers with varying osmolarity. These observation buffers had a constant KCl and Tris concentration (100 and 10 mM, respectively) but varying glucose concentrations (Table 3.9). Osmolarities of the different buffers were measured with an osmometer (Osmomat 3000, Gonotec GmbH). The difference between the osmolarity of the outer solution and the GUV interior is given by ΔOsm (Equation 3.1),

$$\Delta Osm = \frac{V_{OBS}}{V_{Tot}} \cdot Osm_{OBS} + \frac{V_{GUV}}{V_{Tot}} \cdot Osm_{GUV} + \frac{V_{DNA}}{V_{Tot}} \cdot Osm_{DNA} - Osm_{GUV} \tag{3.1}$$

where V_{Tot} is the total volume, and V_{Obs}, V_{GUV}, and V_{DNA} are the volumes of the observation buffer, GUV swelling buffer, and DNA solution, respectively. The other parameters represent the osmolarities of these solutions. The theoretical and actual osmolarity differences are displayed in Table 3.9 as well. Here, the osmolarity difference is positive when the outer solution has a higher osmolarity than the GUV interior, i.e. hypertonic condition. The buffers were prepared in small volumes (1.5 mL), so deviations between the measured and theoretical osmolarities can be explained by small pipetting errors, or improper pipette calibration. After an overnight DNA incubation, the samples were transferred to imaging chambers and imaged.

Solution name	Sugar concentration	Theoretical osmolarity (mOsm)	Theoretical ΔOsm (mOsm)	Measured osmolarity (mOsm)	Resulting ΔOsm (mOsm)
Superhypotonic OBS	67 mM glucose	287	-30	291	-28
Hypotonic OBS	88 mM glucose	308	-15	321	-6
Isotonic OBS	108 mM glucose	328	0	341	9
Hypertonic OBS	129 mM glucose	349	15	369	29
Superhypertonic OBS	149 mM glucose	369	30	392	46
GUV swelling buffer	100 mM sucrose	320	N/A	321	N/A
DNA solution	0	20	N/A	N.A.	N/A

Table 3.9 Solutions used to determine the influence of osmotic conditions on DNA incorporation. Osmolarities were measured in triplicate.

3.8 DNA-mediated vesicle binding

3.8.1 Overview

Unless stated differently, DNA-mediated vesicle binding experiments were performed with 0.1% or 0.5% Atto488 GUVs incubated with 1 µM chol-DNA2-x and 200 nm Atto655 LUVs incubated with 1 µM x-DNA1-chol. Typically, this was done by adding 1 µL 100 µM DNA stock solution to 99 µL vesicles without mixing. Mixing was not found necessary since we generally incubated the samples overnight. Vesicles with DNA were stored in 1.5 mL Eppendorf Tubes ® in the fridge. To test for the level of GUV-LUV binding, 10 µL GUVs with DNA and 10 µL LUVs with DNA were added to 20 µL quenching OBS. They were added to quenching OBS, since this was required for fusion experiments that were often done in parallel. The level of GUV-LUV binding could be determined by quantifying the LUV lipid dye signal on the GUV membrane in fluorescence microscopy images. Imaging settings used to visualise vesicles can be found in Tables 3.4 and 3.5. Samples were closed with lids during imaging to prevent evaporation. An overview of the conditions of the different GUV-LUV binding experiments can be found in Table 3.10. All mixing steps were performed by pipetting the entire solution up and down after adding DNA or LUVs to GUVs.

3.8.2 Timescale and DNA concentration

To determine the timescale of vesicle binding, GUVs and LUVs were incubated separately with their associated ssDNA for 25 minutes. Then, 10 µL GUVs were added to 10 µL LUVs in a 1.5 mL Eppendorf Tube ®, mixed three times, and left to incubate for for 10, 30, 60, 90, or 120 minutes. Afterwards,

Experiment	Type of GUVs	Type of LUVs	DNA concentration (µM)	Mixing DNA	Incubation time DNA	Mixing vesicles	Incubation time vesicles
GUV-LUV binding time	0.5% Atto488 GUVs	Atto655 LUVs	1	Yes (3x)	25 min.	Yes (3x)	10 min., 0.5 hr, 1 hr, 1.5 hr, or 2 hr
DNA concentration	0.5% Atto488 GUVs	Atto655 LUVs	0, 0.25, 0.5, 1, 2.5, or 5	No	Overnight	No	60 min.
Osmotic conditions	0.1% Atto488 GUVs	Atto655 LUVs	1	No	Overnight	Yes (2x)	2 hr

Table 3.10 Overview of the conditions used for the different DNA-mediated GUV-LUV binding experiments. GUV and LUV compositions can be found in Tables 3.4 and 3.5. All LUVs were extruded through a 200 nm pore. DNA or vesicle mixing was performed by pipetting the entire solution up and down.

the vesicles were transferred to a 200 µL microscopy chamber containing 20 µL quenching OBS to determine the level of GUV-LUV binding. The influence of DNA concentration on vesicle binding was determined by doing an overnight incubation of the GUVs and LUVs with 0, 0.25, 0.5, 1, 2.5 or 5 µM of their associated ssDNA. Before the vesicles were added to OBS, 10 µL GUVs and 10 µL LUVs with the same DNA concentration were incubated together in a 1.5 mL Eppendorf Tube ® for one hour to allow for vesicle binding. Then, the vesicles were added to a 200 µL microscopy chamber containing 20 µL quenching OBS to determine the level of GUV-LUV binding at multiple locations in the sample.

3.8.3 Osmotic conditions

To investigate the effect of osmotic conditions on vesicle binding, 0.1% Atto488 GUVs and Atto655 LUVs were first incubated overnight with chol-DNA2-x and x-DNA1-chol, respectively. The next day, the GUVs ans LUVs were added to observation buffers with different osmolarities (Table 3.11). Since LUVs had a lower osmolarity than GUVs, we compensated for that by making observation buffers with higher osmolarity as compared to the DNA incorporation experiment (Table 3.9). The KCl and Tris concentrations of the buffers were again kept constant at 100 and 10 mM, respectively, but the glucose concentration deviated in such a way that comparable osmotic conditions were achieved for the two different experiments. Osmolarities of the observation buffers were measured with the osmometer (Osmomat 3000, Gonotec GmbH). Deviations between the measured and theoretical omsolarities in Table 3.11 can be explained by small pipetting errors or improper pipette calibration. Osmolarities of the vesicle solutions with DNA were determined with Equation 3.2.

$$Osm_{Vesicle+DNA} = \frac{V_{Vesicle}}{V_{Tot}} \cdot Osm_{Vesicle} + \frac{V_{DNA}}{V_{Tot}} \cdot Osm_{DNA} \tag{3.2}$$

The difference in osmolarity between the GUV exterior and interior could then be determined with Equation 3.3.

$$\Delta Osm = \frac{V_{OBS}}{V_{Tot}} \cdot Osm_{OBS} + \frac{V_{GUV+DNA}}{V_{Tot}} \cdot Osm_{GUV+DNA} + \frac{V_{LUV+DNA}}{V_{Tot}} \cdot Osm_{LUV+DNA} - Osm_{GUV} \tag{3.3}$$

In these equations, V_x and Osm_x represent the volumes and osmolarities of the GUVs and LUVs (with DNA), the DNA solutions, the observation buffer, and the total volume. After incubating the vesicles in the different observation buffers for two hours, the samples were transferred to 200 µL microscopy chambers, where the level of GUV-LUV binding was determined.

Solution name	Sugar concentration	Theoretical osmolarity (mOsm)	Theoretical ΔOsm (mOsm)	Measured osmolarity (mOsm)	Resulting ΔOsm (mOsm)
Superhypotonic OBS	68 mM glucose	293	-30	310	-25
Hypotonic OBS	98 mM glucose	323	-15	342	-9
Isotonic OBS	128 mM glucose	353	0	377	8
Hypertonic OBS	158 mM glucose	383	15	415	27
Superhypertonic OBS	188 mM glucose	413	30	452	46
GUV swelling buffer	100 mM sucrose	320	N/A	321	N/A
SUV/LUV buffer	0	260	N/A	247	N/A
DNA solution	0	20	N/A	N.A.	N/A

Table 3.11 Solutions used to determine the influence of osmotic conditions on GUV-LUV binding. Osmolarities were measured in triplicate.

3.9 Bulk fluorescence measurements to develop a content mixing assay

Measurements with the fluorescent dyes sulforhodamine B and HPTS were performed to find a suitable water-soluble dye for a content mixing assay (a method to detect fusion, see Sections 3.9 and 3.10 for more information). A 10 mM sulforhodamine B solution was prepared by diluting 5.8 mg of the dye in 1 mL SUV/LUV buffer. Similarly, a 10 mM HPTS solution was prepared by adding 5.2 mg of the dye to 1 mL SUV/LUV buffer. Both solutions were further diluted to 1, 0.1, and 0.01 mM with SUV/LUV buffer. Since the sulforhodamine B in the 10 mM solution did not fully dilute after adding the dye to the buffer, the solution was vortexed for 10 seconds. To test the ability of DPX to quench HPTS, a 10 mM DPX solution was prepared by adding 4.2 mg DPX to 1 mL OBS. Then, 100 µL of the different dilutions of HPTS were added to 300 µL of the 10 mM DPX solution, resulting in samples containing 2.5, 0.25, 0.025 and 0.0025 mM HPTS and 7.5 mM DPX. Since sulforhodamine B already self-quenches at a concentration of 1 mM [86], there was no need to add a quencher to this dye.

Bulk fluorescence measurements were performed with a plate reader (Infinite M200 Pro, Tecan Group Ltd.) in a dark 384 well plate (MaxiSorp™ 384 well plates, Nunc™). The fluorescence of sulforhodamine B and HPTS (in absence or presence of DPX) was measured with their associated excitation and emission wavelengths (Table 3.2). The z-position was determined from the well containing the first sample, and optimal gain was used. The "Top" mode was selected, and measurements were performed with 25 flashes and an integration time of 20 µs. Since HPTS gave the most promising results because of the large difference in fluorescence in presence or absence of DPX (Section 4.5), the fluorescence measurement of this dye was repeated. In this repeat, a solution of 100 mM HPTS was included by diluting 52.4 mg HPTS in 1 mL SUV/LUV buffer. This solution was vortexed a few minutes to fully dilute the dye. Similar to the first experiment, dilutions of 10, 1, 0.1 and 0.01 mM HPTS were prepared by diluting the 100 mM solution with SUV/LUV buffer. Each HPTS solution was vortexed for 30 seconds before preparing the next dilution. The HPTS samples were again mixed with a 10 mM DPX solution in a 1:3 ratio, resulting in samples containing 7.5 mM DPX and 25, 2.5, 0.25, 0.025 or 0.0025 mM HPTS. To obtain homogeneous samples, the 100 mM HPTS solution was vortexed for 30 second after mixing with DPX, and the other HPTS dilutions were vortexed for 10 seconds after DPX was added. Fluorescence was again measured with the plate reader with the same settings, where each sample was split in three wells and measured twice. To test the stability of the dye, the same samples were measured once more after two days, where the samples were stored in the fridge.

3.10 HPTS-DPX content mixing assay

A content mixing assay with HPTS and DPX was developed, since these compounds were found to be the most useful molecules to detect fusion (Section 4.5). In this assay, LUVs were produced encapsulating 10 mM HPTS. Upon full fusion of LUVs with the GUV, the dye is able to transfer to the GUV lumen. As a result, HPTS should only be visible in the GUV interior upon fusion. We used larger LUVs instead of SUVs (200 and 100 vs 30 nm), since the larger volume of LUVs requires fewer fusion events to detect fusion and to obtain significant membrane growth. By encapsulating HPTS in LUVs,

the fluorescent dye also ended up in the solution that contains these vesicles. To be able to distinguish the HPTS signal from fused GUVs with the HPTS signal from the outer solution, we used a quenching OBS containing 5 mM DPX was used (Table 3.3). The DPX concentration of the quenching OBS was chosen in such a way that the HPTS and DPX concentrations were equal when the vesicles were added to quenching OBS, assuming that DPX successfully quenches HPTS when they have the same concentration.

GUVs and LUVs were generally added to quenching OBS with a volume ratio of 1:1:2, where the experiment testing the effect of vesicle mixing order and KCl concentration on fusion is the only exception (Subsection 3.11.7). Here, GUVs and LUVs were added to quenching OBS in a 1:1:8 volume ratio for the samples with a lower KCl concentration. Typical volumes used were 20 µL quenching OBS, 10 µL GUVs and 10 µL LUVs, or 25 µL quenching OBS, 25 µL GUVs and 25 µL LUVs. The vesicles were imaged in 200 µL microscopy chambers. Before the vesicles were mixed with OBS, they were generally incubated with each other for 60-80 minutes, since this greatly enhanced the fraction of fused vesicles compared to directly adding vesicles to OBS (Subsection 4.7.6).

3.11 DNA-mediated vesicle fusion

3.11.1 Overview

To measure the fusion rate under a variety of different conditions (i.e. the fraction of vesicles fused in a sample population), DNA-mediated fusion experiments were performed with the ssDNAs incorporated in the same way as in the GUV-LUV binding experiments: chol-DNA2-x was incorporated in the GUVs, and x-DNA1-chol was incorporated in the LUVs. An overview of the conditions of the different DNA-mediated fusion experiments performed can be found in Table 3.12. Fusion was detected with the HPTS-DPX content mixing assay (Section 3.10). Imaging settings used to detect the vesicles depends on the vesicle type and can be found in Tables 3.4 and 3.5. To detect fusion, the HPTS imaging settings were used (Table 3.6), of which the experiment-specific exposure times can be found in Table 3.12. For all experiments, except the one where the influence of LUV size on fusion rate was investigated, samples were closed with lids during measurements to prevent evaporation.

To avoid a selection bias towards GUVs with internal HPTS (whose fluorescent spectra partly overlap), the samples were generally observed in the LUV channel, and images were made in the GUV, LUV and HPTS channels. There are a few exceptions for this way of detecting fused vesicles. The first one was is the experiment where the influence of GUV lipid dye was tested (Subsection 3.11.6). Here, unlabelled vesicles were observed using brightfield microscopy. Another exception is the experiment where the effect of vesicle mixing order on fusion was tested (Subsection 3.11.7). Here, fusion was detected by looking around in the HPTS channel for the samples where GUV and LUVs were directly added to quenching OBS, since these samples had a way lower fusion rate. For control samples of DNA-mediated vesicle fusion, where no GUV-LUV binding was expected, the samples were observed in the GUV channel, and images were made in the GUV, LUV and HPTS channel. In this case, we experienced no issues with fluorescence crosstalk between the GUV and HPTS channel, since the number of vesicles with internal HPTS as well as the internal HPTS signal was relatively low.

Experiment	Type of GUVs	Type of LUVs	DNA concentration (μM)	Mixing DNA	Incubation time DNA	Incubation time vesicles (min.)	Mixing vesicles	HPTS exposure time (ms)
DNA concentration	0.5% Atto488 GUVs	Atto655 LUVs	0, 0.25, 0.5, 1, 2.5, or 5	No	Overnight	60	No	50
Osmotic shock	0.5% Atto488 GUVs	Atto655 LUVs	1	Yes (3x)	15 min.	70	Yes (3x)	25
LUV size	Unlabelled GUVs	RhoPE LUVs (100 + 200 nm)	1	No	1.5 hr (GUVs + 100 nm LUVs) or 1 hr (200 nm LUVs)	70	No	100
GUV lipid composition and formation method	0.5% Atto488 (Gel-assisted swelling & eDICE) + Physiologically relevant + PE-containing GUVs	Atto655 LUVs	1	Yes (3x)	15 min.	70	Yes (3x)	25
GUV lipid dye	Unlabelled + 0.5% Atto488 + 0.5% Atto655 GUVs	Unlabelled LUVs	1	No	Overnight	70	No	100 (no GUV dye) or 50 (other samples)
Vesicle mixing order and KCl concentration	0.1% Atto488 GUVs (with or without KCl)	Atto655 LUVs	2.5	No	Overnight	0 or 70-80	Only for sample without KCl and without vesicle incubation (2x)	10 (sample 4) or 100 (other samples)

Table 3.12 Overview of the conditions used for the different GUV-LUV fusion experiments. GUV and LUV compositions can be found in Tables 3.4 and 3.5. All GUVs were prepared by gel-assisted swelling, except for the eDICE vesicles tested in the "GUV lipid composition and formation method" experiment. GUVs without KCl in the "Vesicle mixing order and KCl concentration" experiment were prepared with swelling buffer containing 200 mM sucrose, 10 mM Tris (pH 7.4) and no KCl. All LUVs were extruded through a 200 nm pore, except for the "LUV size" experiment. Incubation time vesicles indicates the time that GUVs and LUVs were incubated with each other before they were added to quenching OBS. Mixing vesicles indicates if vesicles were mixed with quenching OBS after this buffer was added. DNA or vesicle mixing was performed by pipetting the entire solution up and down.

3.11.2 DNA concentration

To test the influence of DNA concentration on vesicle fusion, 0.5% Atto488 GUVs and 200 nm Atto655 LUVs were incubated overnight with 0, 0.25, 0.5, 1, 2.5 or 5 µM of their associated ssDNA. The next day, 10 µL GUVs and 10 µL LUVs with equal amounts of DNA were incubated together for 60 minutes in a 1.5 mL Eppendorf Tube ®. After incubation, the vesicles were added to 20 µL quenching OBS in 200 µL microscopy chambers and vesicles were imaged in the GUV, LUV and HPTS channels.

3.11.3 Osmotic shock

During the GUV-LUV mixing step of fusion experiments, GUVs membrane tension was temporarily increased through an osmotic shock ($\Delta_{Osm} \approx$ -30 mOsm, see Subsection 4.7.2 for a more elaborate explanation). To test the effect of this osmotic shock on fusion rate, 0.5% Atto488 GUVs that underwent an osmotic shock (hypotonic conditions) were compared to 0.5% Atto488 GUVs that experienced approximate isotonic conditions during the whole experiment. For the experiment, 200 nm Atto655 LUVs were used. The osmotic shock sample was prepared as described in Section 3.10. For the other sample, 13.16 µL 1 M glucose was added to 250 µL LUVs with DNA, increasing its osmolarity from 260 to 310 mOsm. As a result, the difference in osmolarity between the GUV interior and exterior was only ~5 mOsm when GUVs were mixed with LUVs. To test the effect of GUV membrane tension on fusion rate, 25 µL GUVs were incubated with 25 µL LUVs (with or without osmotic shock) after DNA incubation of 15 minutes. For both shock and non-shock conditions, we added two controls to test for membrane porosity: one with DNA only on GUVs and in total absence of DNA. This was done by mixing 25 µL GUVs (with or without 1 µM DNA) with 25 µL LUVs without DNA (with or without osmotic shock) in 1.5 mL Eppendorf Tubes ®.

For all samples described in this subsection, 50 µL quenching OBS was added after 70 minutes of vesicle incubation. The samples were then mixed three times by pipetting the solution up and down. After this, 40 µL of each sample was added to a 200 µL microscopy chamber to detect fusion.

3.11.4 LUV size

The influence of LUV size on fusion was investigated by comparing the fusion rate of 100 with 200 nm LUVs. This was done by incubating 1 µM x-DNA1-chol with 200 nm RhoPE LUVs for 1 hr, 1 µM x-DNA1-chol with 100 nm RhoPE LUVs for 1.5 hr, and 1 µM chol-DNA2-x with unlabelled GUVs for 1.5 hr. Then, 25 µL LUVs were incubated with 25 µL GUVs for 70 minutes. After vesicle incubation, GUVs and LUVs were added to 200 µL microscopy chambers containing 50 µL quenching OBS. Then, fusion could be detected by taking images in the GUV, LUV and HPTS channels.

3.11.5 GUV lipid composition and formation method

To test if the fusion protocol is compatible with other vesicle conditions, we determined the fusion rate of several GUV lipid compositions and GUV formation methods. For this, 1 µM chol-DNA2-x was incubated with three GUV types that were prepared with gel-assisted swelling (0.5% Atto488, physiologically relevant, and PE-containing GUVs), and one type that was formed with eDICE (0.5% Atto488). eDICE vesicles were prepared as described in Lambert [29], where it was ensured that the inner and outer buffer conditions were similar to vesicles formed through gel-assisted swelling. Since this required the vesicles to be diluted, the eDICE vesicles had a lower vesicle density than the GUVs formed by gel-assisted swelling. 200 nm Atto655 LUVs were incubated with 1 µM x-DNA1-chol. After mixing the vesicles with DNA three times by pipetting the solutions up and down, and a waiting step of 15 minutes, 25 µL of each GUV sample was added to 25 µL LUVs. As a control for non-specific fusion, 25 µL of each GUV sample with DNA was added to 25 µL LUVs without DNA. After 70 minutes, 50 µL quenching OBS was added to each sample, and each sample was mixed three times. Then, 40 µL of each sample was added to a 200 µL microscopy chamber to determine the fusion rate of the different samples.

3.11.6 GUV lipid dye

The influence of GUV lipid dye on fusion rate was tested by preparing unlabelled, 0.5% Atto488 and 0.5% Atto655 GUVs. The LUVs used in this experiment contained no lipid dye and were extruded through a 200 nm pore. The vesicles were incubated overnight with 1 µM of their associated ssDNA without mixing. The next day, 10 µL of the different GUV samples was incubated with 10 µL LUVs in 1.5 mL Eppendorf Tubes ®. After 70 minutes, 20 µL quenching OBS was added, and the samples were transferred to 200 µL microscopy chambers to detect fusion.

3.11.7 Vesicle mixing order and KCl concentration

Through experimental observations, we observed an effect of mixing vesicle order on fusion efficiency. To test the effect of vesicle mixing order on fusion rate in a single experiment, GUVs and LUVs were either incubated with each other for 70-80 minutes before quenching OBS was added (i.e. the working fusion protocol), or GUVs and LUVs were directly added to quenching OBS (i.e. the ineffective fusion protocol). For this experiment, 0.1% Atto488 GUVs and 200 nm Atto655 LUVs were used. The GUVs and LUVs were incubated overnight with their associated ssDNAs. This experiment was done in parallel with an experiment where the effect of KCl concentration on GUV-LUV binding and fusion was investigated. Part of the GUVs were therefore prepared with a GUV swelling buffer containing no KCl, 200 mM sucrose and 10 mM Tris (pH 7.4), instead of the GUV swelling buffer used for other experiments (Table 3.3). The quenching OBS used to observe GUVs without KCl neither contained KCl. Instead, it contained 200 mM glucose, 10 mM Tris (pH 7.4) and 5 mM DPX. Since all LUVs did contain 100 mM KCl, the samples with a lower KCl concentration had a final KCl concentration of 10 mM after adding 10 µL GUVs and 10 µL LUVs to 80 µL quenching OBS. These conditions resulted in three samples (Table 3.13).

Sample no.	Sample composition	KCl concentration (mM)	Incubation time vesicles (min.)
1	10 µL GUVs (no KCl) + 10 µL LUVs (KCl) + 80 µL quenching OBS (no KCl)	10	0
2	10 µL GUVs (no KCl) + 10 µL LUVs (KCl) + 80 µL quenching OBS (no KCl)	10	70
3	25 µL GUVs (KCl) + 25 µL LUVs (KCl) + 50 µL quenching OBS (KCl)	100	80

Table 3.13 Sample compositions, KCl concentrations and vesicle incubation times of the different samples used in the experiment assessing the effect of vesicle mixing order and KCl concentration. The vesicle incubation time is the time that GUVs and LUVs were mixed with each other before quenching OBS was added.

Samples were observed in 200 µL microscopy chambers. For samples where the GUVs and LUVs were directly added to quenching OBS, fused vesicles were detected by looking around in the HPTS channel. For samples where GUVs and LUVs were added to quenching OBS after vesicle incubation, fused vesicles were observed by looking around in the LUV channel. For each sample, images were made in the GUV, LUV, and HPTS channels. For the sample of the ineffective fusion protocol, we looked around in the sample for fused vesicles in the HPTS channel, whereas for the working fusion protocol, we looked around in the LUV channel.

3.12 Image analysis

The obtained images were analysed using the DisGUVery software [87]. Image analysis was generally performed by Lennard van Buren, and plots were made by Tom Aarts from the processed data. After a smoothing and edge enhancement step, GUVs were detected through the signal of their lipid dye with the circular Hough detection algorithm [88]. Data of the different imaging channels were subsequently extracted both with angular and radial profiles. The angular profile formed a ring around the GUV

membrane of 50 pixels wide, and divided this ring in 72 angular slices. To determine the DNA or LUV signal on the GUV membrane in each slice, we took the maximum signal in that slice. The average signal on the membrane of an individual GUV was then taken to be the median of all maxima along the contour. The radial profile was calculated by integration of pixel intensities in concentric rings of 5 pixels wide, starting around the centre of the GUV and expanding until the ring was just outside of the GUV. The radial profile was used to determine the DNA and LUV background signal, where the mean signal value of the outer most ring was taken, thus corresponding to the signal just outside the vesicle. This background signal was subsequently subtracted from the DNA or LUV signal measured on the GUV membrane. The radial profile was also used to determine the HPTS ratio, where the mean signal of the inner most ring (in the centre of the GUV) was divided by the signal of the outer most ring (just outside of the GUV).

4

Results

In this study, we investigated fusion of GUVs with LUVs using a semi-quantitative fluorescence microscopy approach. We focused on fusion mechanisms based on DNA and tension. We also performed experiments with charge-mediated fusion, but observed a low level of fusion and high levels of vesicles sticking to each other with this approach (Appendix A). We observed the localisation of LUVs on GUV membranes (Section 4.1) and performed DLS measurements to determine LUV sizes and stability (Section 4.2). Next to this, we studied how ssDNA inserts into GUV membranes as a function of environmental factors (Section 4.3) and how these factors affect DNA-mediated GUV-LUV binding (Section 4.4). We also performed bulk fluorescence experiments to find a suitable candidate for a content mixing assay (Sections 4.5 and 4.6). Finally, we explored the parameter spaces of DNA and tension-mediated GUV-LUV fusion, and made estimates on the amount of membrane growth we possibly obtained (Section 4.7).

4.1 Vesicle visualisation

In this thesis, GUV lipid dyes of microscopy images will be visualised in red, whereas LUV lipid dyes will be visualised in cyan. We were able to successfully visualise LUV localisation on the GUV membrane by coupling both vesicle types with a complementary ssDNA (Figure 4.1). In Section 4.4, we will show that there was little to no fluorescent crosstalk from the GUV lipid dye used into the LUV channel. Due to the small sizes of the LUVs (~100-200 nm), which is below the diffraction limit, we were not able to resolve them individually. Instead, we could only observe a population of LUVs that were bound to the GUV membrane, and a homogeneous or speckled background signal. To determine the size of individual LUVs, other techniques such as DLS are required (Section 4.2).

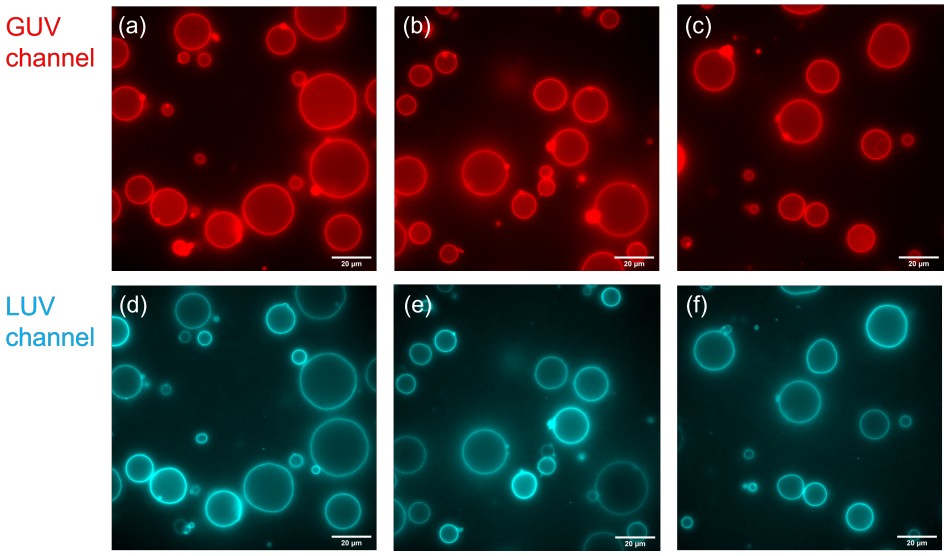


Figure 4.1 Visualisation of 0.5% Atto488 GUVs (a-c) and 200 nm Atto655 LUVs binding to the same GUVs (d-f) (complete lipid compositions can be found in Tables 3.4 (GUVs) and 3.5). Scale bars indicate 20 µm.

4.2 LUV and SUV characterisation

4.2.1 Vesicle size and stability

LUVs were formed by extruding multilamellar vesicles through polycarbonate membranes with a pore size equal to the desired vesicle size (Subsection 3.3.2). To verify the size of LUVs after extrusion, we performed DLS measurements. The results show particle size distributions (PSDs) based on the measured intensity (Figure 4.2a) as well as the number of vesicles (Figure 4.2b). Due to the difference in measured diameters between the PSDs, we only use DLS results as a semi-quantitative measurement. For both PSDs, we clearly see that the measured diameter decreases with the pore size used for vesicle extrusion. Since DLS measurements are based on light scattering, it is generally recommended to display DLS results in terms of measured intensity [89]. For nanoparticles (1 < d < 100 nm), however, it can be more suitable to display the size distributions in terms of number of particles [90]. This is because small particles scatter less light compared to larger particles. Therefore, smaller particles can be under-represented in the intensity PSD [91]. Corresponding to this, the number PSDs show smaller vesicle diameters than the intensity PSDs (Figure 4.2ab).

We also assessed LUV stability over time (Figure 4.2cd).[1] Since we assessed this for 100 nm LUVs, we only show the intensity PSDs. For the sample of one week old, we observed broader distributions of particle sizes compared to fresh vesicles, potentially indicating vesicle aggregation. For 30 nm SUVs, used for charge-based experiments, we observed a broader size distribution for vesicles of one week old as well (Figure A.1). We therefore decided to prepare LUVs and SUVs fresh for each experiment independent of membrane composition used.

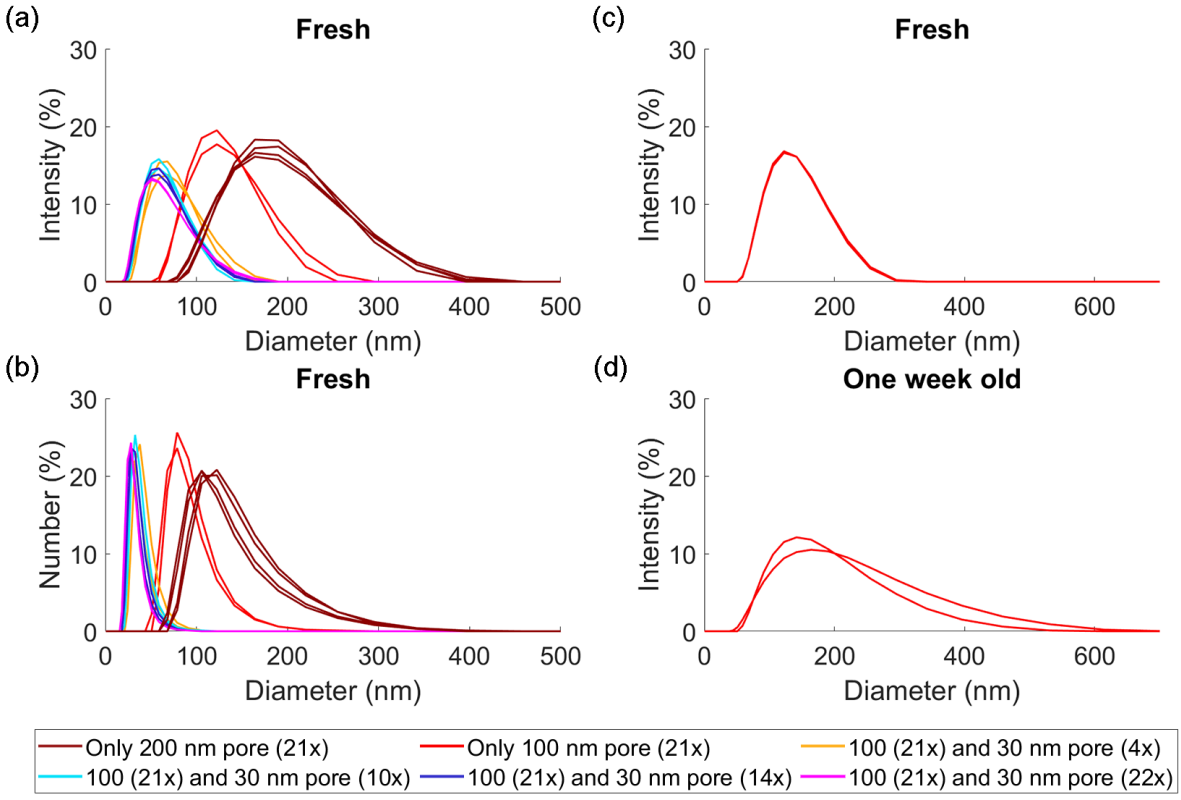


Figure 4.2 DLS measurements to verify sizes of fresh LUVs (a and b), and stability of fresh (c) and one week old (d) LUVs extruded through 100 and 200 nm pores. Intensity PSDs (a, c, and d) as well as number PSDs are displayed (b). LUVs had the following lipid compositions: 100 mol% DOPC (a and b) and 60 mol% DOPC + 40 mol% DOTAP (c and d). Each curve represents one measurement, that was performed on a single sample (30 nm SUVs and 100 nm LUVs) or on two different samples (200 nm LUVs).

[1] This was only done for 100 nm 40% TAP (charged) LUVs.

4.2.2 Number of extrusion steps

Repeated LUV extrusion is a cumbersome and error-prone process that often involved sample leakage in our case. We therefore investigated the size of LUVs after varying numbers of extrusion steps (Figure 4.2ab). Here, we found that an extrusion of four times is sufficient to drastically decrease the vesicle size from 100 to 30 nm, and no further great reduction of the vesicle size was obtained after extruding the solution 18 more times. The width of the peak pushed four times through the 30 nm pore is a bit broader than the peak pushed 22 times[2] through the pore (mean full width at half maximum (FWHM) of intensity PSD $\approx$ 68 nm for 4 pushes, mean FWHM of intensity PSD $\approx$ 63 nm for 22 pushes), indicating that the vesicle size distribution became somewhat narrower after more pushes.

Since the difference in peak width is relatively small, we conclude that four extrusion steps are sufficient to obtain vesicles with a narrow PSD and with vesicle sizes close to the minimum, which deviates from the general performance of 21 extrusion steps [e.g. in 30, 62]. For charged vesicles, we found a clearly narrower PSD after 21 extrusion steps compared to 9 extrusion steps (Figure A.1). We detected low fusion rates using 30 nm SUVs (Appendix A), probably caused by their low amount of HPTS contents delivered per fusion event (see Section 4.6). We therefore only used 100 and 200 nm LUVs for DNA and tension-mediated fusion.

[2] Deviates from the usual odd number of extrusions steps for experimental reasons (Subsection 3.3.2), but still resulted in a clean sample containing only membranous structures that passed through the polycarbonate membrane.

4.3 DNA incorporation

4.3.1 Indirect control to detect DNA incorporation

Before we endeavoured to achieve vesicle fusion through DNA hybridisation, we first assessed if we were able to incorporate the ssDNA in our GUVs. To test this, we would ideally have used an ssDNA with a cholesterol tag on one end, and a fluorophore on the other end. In this way, the hydrophobic part of the cholesterol group would slide into the GUV membrane, while the fluorophore on the other end of the ssDNA would allow us to observe it. If the ssDNA signal would localise on the GUV membrane, this would mean that it is successfully incorporated into the vesicle. Since we were not able to order an ssDNA with this design, we tested the level of DNA incorporation with an indirect control (Figure 4.3a). In this experiment, we added chol-DNA2-x to our GUVs, together with the complementary and fluorescently labelled x-DNA1-Atto488 (Table 3.7). With this indirect control, Atto488 signal on the GUV membrane meant that the ssDNAs were both incorporated into the membrane and bound to their complementary strands. In Figure 4.3a, all ssDNAs are incorporated into the membrane, and all ssDNAs are hybridised with their complementary strand. In reality, at least part of the ssDNAs that are fluorescently labelled are not bound to an ssDNA that is incorporated in the GUV membrane. We know this from the background signal observed when using this fluorophore, which is substantially increased for higher DNA concentrations (Figure 4.7b).

When no ssDNA was added, we observed no DNA signal, meaning that there was no spectral crosstalk in the DNA channel (Figure 4.3b). If only fluorescent ssDNA was added, we only observed a DNA background signal but no DNA signal on the GUV membrane, which means that there was no non-specific binding of fluorescent ssDNA to GUV membranes (Figure 4.3c). Finally, if we added both types of ssDNA, we observed a clear DNA signal on the GUV membrane, indicating both DNA incorporation and DNA hybridisation. These observations allowed us to proceed with experiments where we addressed the following research questions: what is the timescale of DNA incorporation, and do DNA concentration and membrane tension have an effect on DNA incorporation?

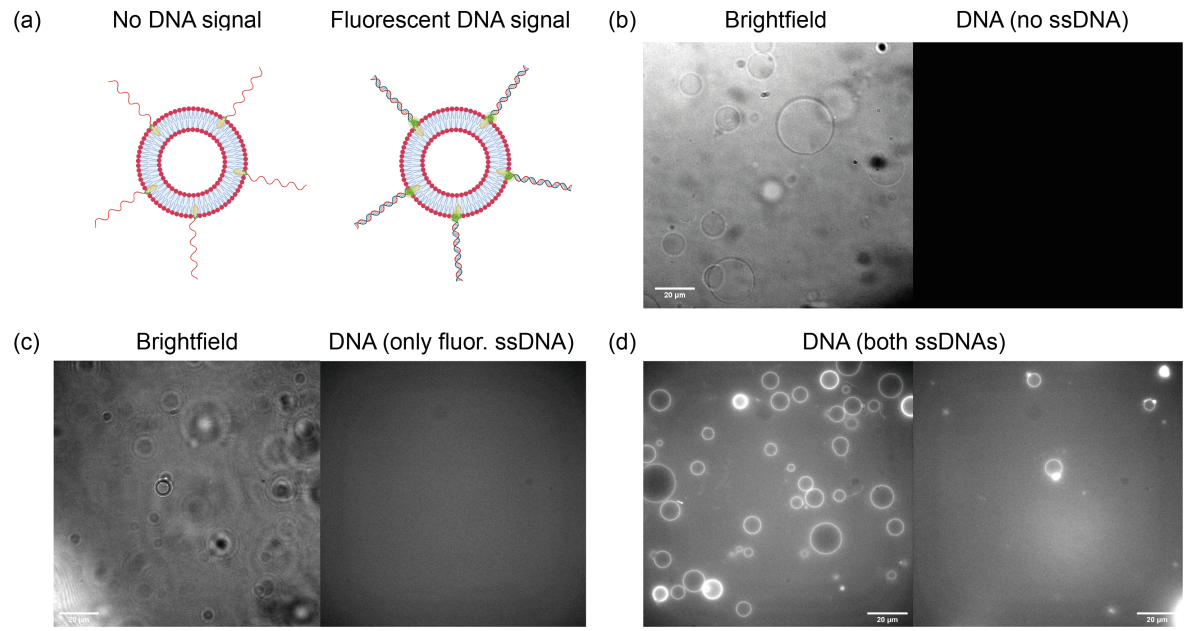


Figure 4.3 Indirect control to asses if the DNA got incorporated in the GUV membrane. (a) Schematic representation of the experiment, where a fluorescent DNA signal is only visible when both ssDNAs are added (Figure made with BioRender). (b) No DNA signal was visible in the DNA channel when no ssDNA was added. (c) When the fluorescent ssDNA was added, a DNA background signal was visible, but no DNA signal on the GUV membranes. (d) When both ssDNAs were added, a clear DNA signal was observed on the GUV membranes. A DNA concentration of 1 µM was used. Microscopy images were made in the brightfield and DNA-Atto488 channels when no DNA incorporation was expected (Subsection 3.5.3), and only in the DNA-Atto488 channel when DNA incorporation was expected. Scale bars indicate 20 µm.

4.3.2 GUV-DNA crosstalk

In experiments where the incorporation time of DNA (Subsection 4.3.3) and the effect of osmotic conditions on DNA incorporation (Subsection 4.3.5) were studied, we used GUVs labelled with the lipid dye Atto655. This lipid dye was used to be able to detect the GUVs. We verified that there was little to no fluorescent bleed-through from Atto655 into the fluorescent DNA channel (Figure 4.4). Therefore, fluorescent DNA signal could be used for semi-quantitative analysis.

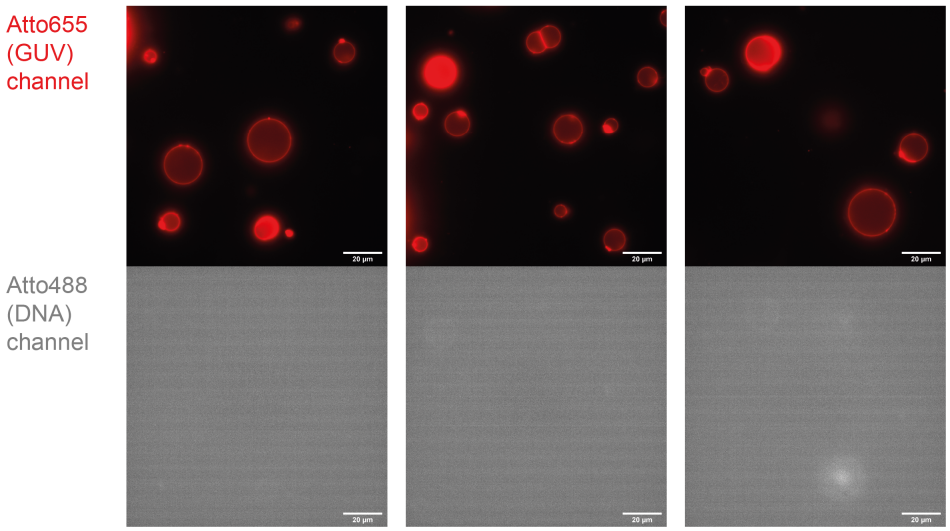


Figure 4.4 Control of fluorescence crosstalk of Atto655 (GUV lipid dye) into the Atto488 channel (DNA fluorophore). Images above each other are taken of 0.01% Atto655 GUVs without DNA using the GUV-0.1%Atto655 and DNA-Atto488 imaging settings (Subsection 3.5.3). Little to no crosstalk was observed for all images. Scale bars indicate 20 µm.

4.3.3 DNA incorporation time

The first question we aimed to address is the following: what is the timescale of DNA incorporation? For this, we measured the DNA signal on GUV membranes at multiple time points after adding both ssDNAs (Figure 4.5). The first four time points were observed in a single microscopy chamber (sample 1), and the level of DNA incorporation after an overnight incubation was assessed in another chamber (sample 2). By visual inspection, it looks like both the level of DNA incorporation and background signal decreased from t = 5 min. to t = 0.5 hr. Next to this, it seems like these values decreased for the first four time points, but that they were comparable between an incubation time of 5 minutes and an overnight incubation. Quantification of the DNA signal at the GUV membrane, however, revealed that its average value was comparable for the different time points (Figure 4.6). A small fraction of the vesicles show a negative background-subtracted DNA signal, meaning that the background signal was higher than the DNA signal on the membrane. We found no significant difference between level of DNA incorporation for the different time points (one-way ANOVA, $p > 0.05$), and can therefore conclude that a short incubation time of 5 minutes is sufficient to achieve the maximum level of DNA incorporation.

We observed a decrease in the background signal over time for sample 1, which we think is due to sequestration of unincorporated DNA to the sample chamber walls. This effect should be taken into account when performing experiments with a longer duration. We hypothesise that the larger number of vesicles with a lower DNA signal for the overnight sample compared to the t = 5 min. sample is a background correction artefact. For background correction, the average intensity of the ring just outside the vesicle is subtracted from the DNA signal on the membrane (Section 3.12). Since the vesicle density of the overnight sample was higher than the t = 5 min. sample (Figure 4.5), the ring outside of the vesicle could contain intensity values of other vesicles when multiple vesicles were touching each other, thereby increasing the subtracted background value.

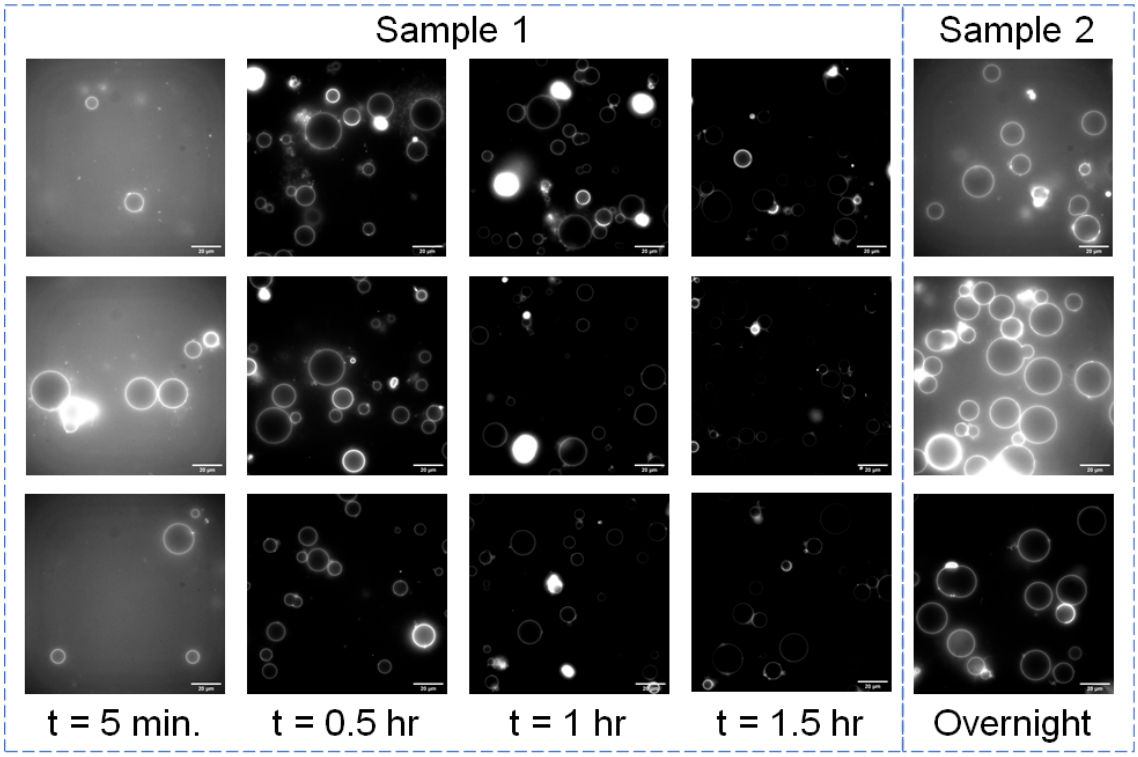


Figure 4.5 Experimental observation of DNA incorporation in GUV membranes at different time points. The first four time points are observed in a single microscopy chamber (sample 1), the overnight sample is observed in another microscopy chamber (sample 2). A DNA incubation time of 5 minutes shows similar level of DNA incorporation compared to an overnight incubation. The decreasing background signal of sample 1 is thought to be due to ssDNAs binding to the glass surface of the microscopy chamber (see main text). Scale bars indicate 20 μm.

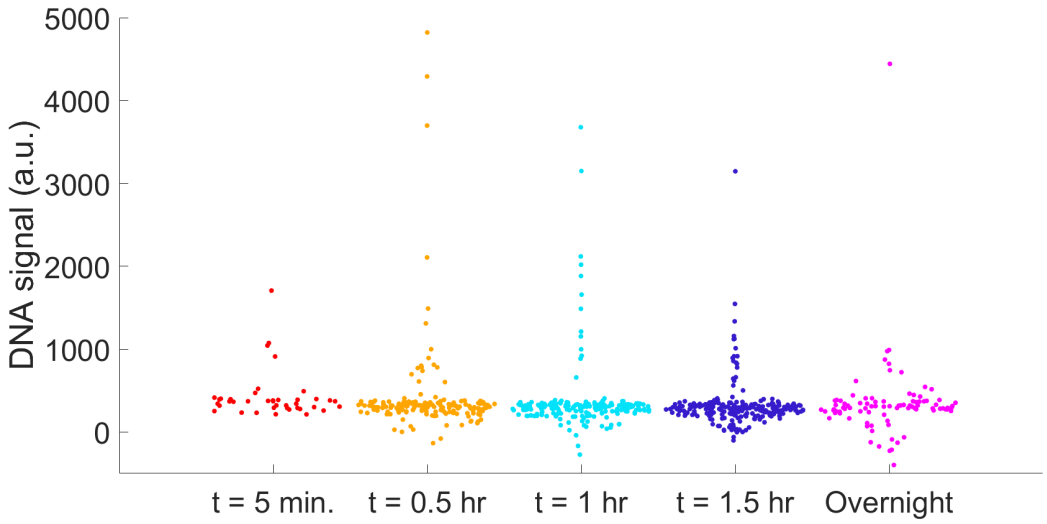


Figure 4.6 Quantification of DNA incorporation in GUV membranes at different time points: t = 5 min. (n = 40), t = 0.5 hr (n = 164), t = 1 hr (n = 200), t = 1.5 hr (n = 235), and overnight (n = 90) using 1 μM DNA. DNA signal indicates the DNA signal on the GUV membrane with the local DNA background signal subtracted. Each data point of the swarm plot represents a single vesicle.

4.3.4 DNA concentration

To test the influence of DNA concentration on DNA incorporation, we added different amounts of the cholesterol-tagged and fluorescently labelled ssDNAs to GUVs (0, 0.25, 0.5, 1, 2.5, or 5 µM). The concentrations of the two different ssDNAs were equal to each other in each sample. After an overnight incubation, we determined the signal from the fluorescent DNA on the GUV membrane. We observed more heterogeneity in DNA signal for the highest DNA concentration (5 µM) compared to the lowest DNA concentration (0.25 µM) (Figure 4.7).

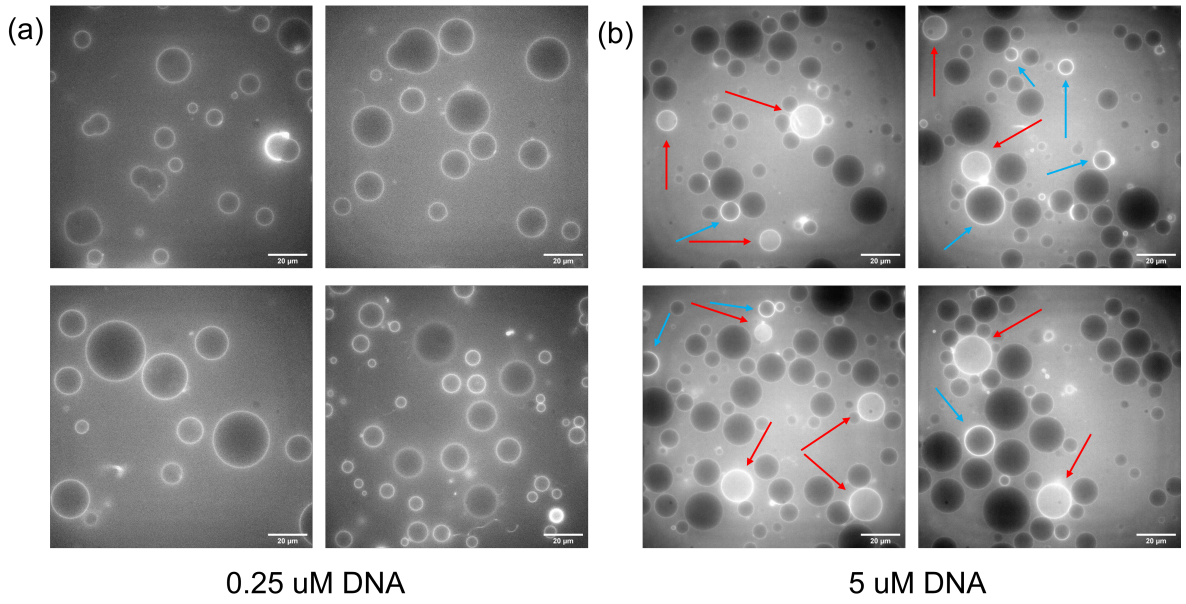


Figure 4.7 Comparison of DNA incorporation for 0.25 (a) and 5 (b) µM DNA. The sample with 5 µM DNA shows more heterogeneity in DNA signal on the GUV membrane, causing a larger standard deviation in DNA signal on the GUV membrane (Figure 4.8). The GUVs with a higher DNA signal often showed an internal DNA signal as well (red arrows), but not in all cases (blue arrows). Scale bars indicate 20 µm.

The vesicles showing a higher DNA signal on the GUV membrane often showed an internal DNA signal as well (Figure 4.7, red arrows). Since this indicates that DNA was also present inside of the vesicle, we believe that the higher DNA signal on the membrane was caused by DNA incorporation in both membrane leaflets. We hypothesise that the inflow of DNA was especially observed for higher DNA concentrations due to an increased membrane porosity, possibly caused by strong DNA-DNA interactions disrupting lipid interactions in the bi-layer. The vesicles with a higher level of DNA incorporation frequently showed membrane fluctuations as well (Video S1, available online). Since osmotic pressure is released for porous vesicles, we believe that these fluctuations were also caused by increased membrane porosity. In the cases where a high DNA signal on the GUV membrane was observed but no internal DNA signal (Figure 4.7, blue arrows), we expect that the GUVs were less porous than the GUVs showing an internal DNA signal. As a result, all DNA that entered the interior of GUVs got incorporated into the inner membrane leaflet.

The semi-quantitative population analysis for the different DNA concentrations is displayed in Figure 4.8. As expected, the measured DNA signal increased with DNA concentration. We found no significant difference in DNA incorporation between the two highest DNA concentrations (one-way ANOVA, $p = 0.66$). This indicates that the level of DNA incorporation was plateauing for the higher DNA concentrations, suggesting DNA saturation of the vesicles. This result also suggests that, especially for higher DNA concentrations, not all DNA got inserted. The fact that we observed a background signal using 0.25 µM DNA (Figure 4.7a) suggests that unincorporated DNA was present at lower DNA concentrations as well. We observed a larger spread in DNA signal for higher DNA concentrations. We hypothesise that this was caused by double-leaflet DNA incorporation. In addition, the larger spread can be due to a lower signal-to-background ratio for the samples with higher DNA concentrations.

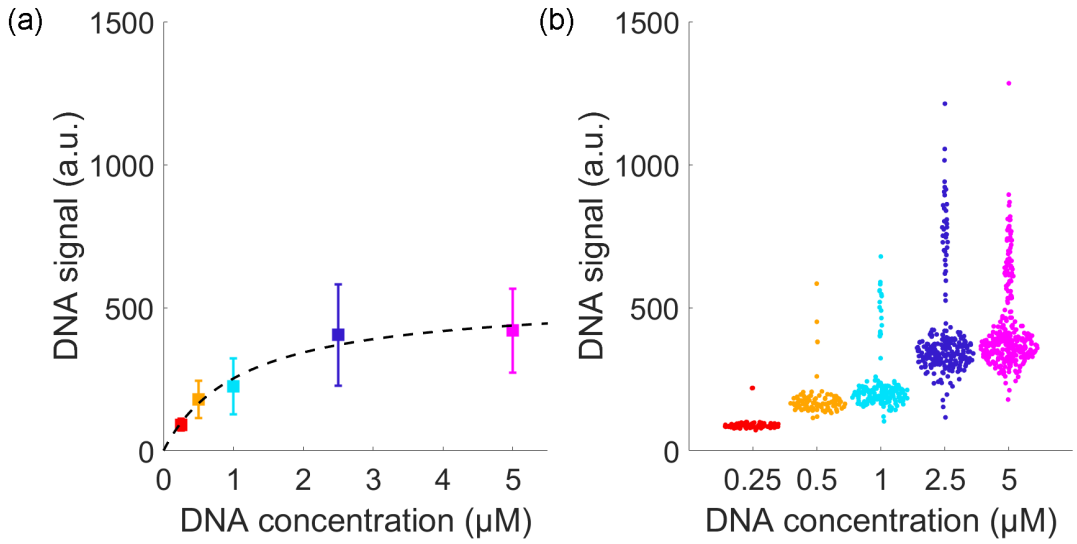


Figure 4.8 The effect of DNA concentration on DNA incorporation. DNA incorporation in GUV membranes was measured for 0.25 (n = 83), 0.5 (n = 91), 1 (n= 171), 2.5 (n = 236) and 5 (n = 328) µM DNA. (a) Markers indicate mean level of DNA incorporation averaged over the entire vesicle populations, and error bars indicate standard deviations. The individual data points are fitted with a Langmuir equation (K = 0.89 μM^{-1}, R^2 = 0.97) using the fitAdsorptionIsotherm MATLAB package [92]. (b) Swarm plot of the same data, where each data point represents a single vesicle.

We can describe this DNA saturation with a Langmuir adsorption model [93]. This model describes the adsorption of molecules to a surface based on the number of available positions for binding, and has been used previously to describe the adsorption of DNA to lipid monolayers [94] and SLBs [95, 96]. Since this process is similar to DNA incorporation into vesicles, we fitted the Langmuir equation with the average values of DNA incorporation in Figure 4.8a (K_D = 1.12 µM, R^2 = 0.97). Here, the level of DNA incorporation is described by Equation 4.1,

$$N = \frac{N_0 \cdot P}{K_D + P} \tag{4.1}$$

where N is the fluorescent signal of DNA on the GUV membrane, N_0 is the fluorescent signal on the DNA at maximum incorporation, K_D = (k_{off}/k_{on}) is the dissociation constant, and P is the DNA concentration [92].[3] The dissociation constant K_D is the the DNA concentration where the level of DNA incorporation is half the saturation value N_0, and thereby indicates the binding affinity between the DNA and the GUV membrane.

From the results, we learn that a DNA concentration of 2.5 µM is sufficient to get close to the maximum level of DNA incorporation. However, we will later show that 1 µM DNA results in more GUV-LUV binding (Subsection 4.4.2) and GUV-LUV fusion (Subsection 4.7.1). For the majority of the experiments, we therefore used a DNA concentration of 1 µM. Adding more DNA will not increase DNA incorporation much further, and might even cause experimental side-effects due to a higher amount of free DNA in solution. The process of DNA binding in both membrane leaflets was mainly observed for the higher DNA concentrations, possibly overestimating the level of DNA incorporation at these concentrations. This would mean that the actual value of the equilibrium constant K_D is lower (i.e. half the maximum level of DNA incorporation is achieved for a lower DNA concentration), resulting in a stronger binding affinity than displayed here.

[3] Here, we assume that the background-subtracted fluorescent signal on the GUV membrane increases linearly with the number of molecules adsorbed to the GUV membrane.

4.3.5 Osmotic conditions

Increasing membrane tension has been reported to promote vesicle fusion (Section 2.7) [30]. We aim to adopt this approach to facilitate membrane growth for our vesicles. In this approach, membrane tension is increased by changing the osmotic conditions of the solution in which the vesicles are dispersed. To test if adapting membrane tension has an effect on DNA incorporation, we varied the osmolarity of the GUV outer solution and studied its influence on DNA incorporation. Here, we studied samples from superhypotonic (ΔOsm = -28 mOsm) to superhypertonic (ΔOsm = 46 mOsm) conditions (Figure 4.9), where the osmotic difference ΔOsm indicates the difference in osmolarity between the vesicle outer solution and GUV interior. From the results, we can appreciate that osmotic conditions have no great effect on DNA incorporation. We indeed found no significant difference in DNA signal between all osmolarity differences tested (one-way ANOVA, $p > 0.05$).

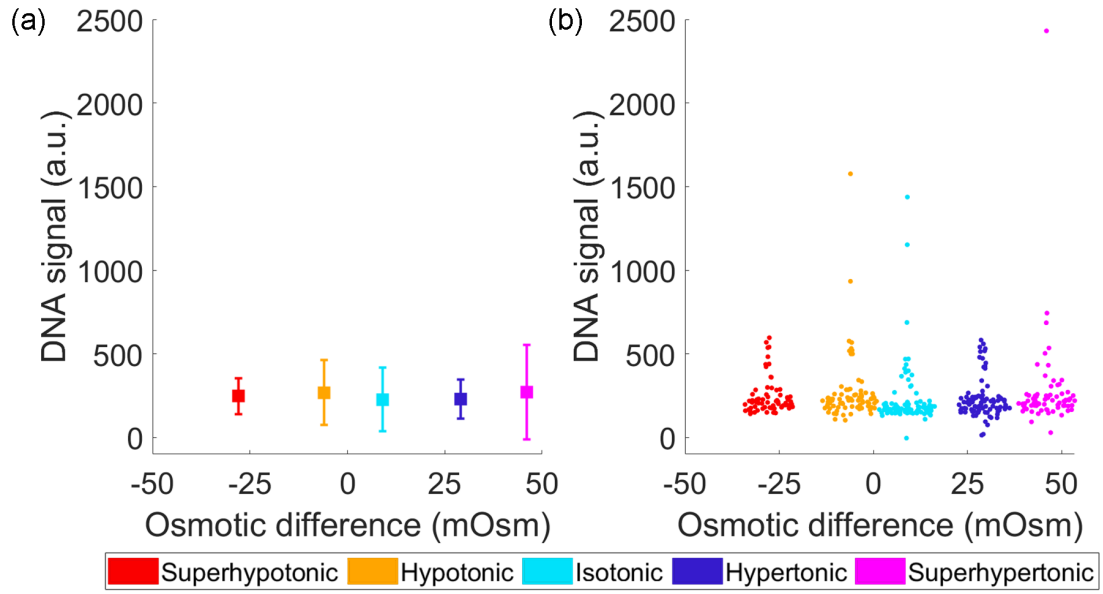


Figure 4.9 The effect of varying osmotic conditions on DNA incorporation. Background-subtracted DNA signal for superhypotonic (n = 66), hypotonic (n = 81), isotonic (n = 90), hypertonic (n = 91), and superhypertonic (n = 72) conditions is displayed. (a) Markers indicate mean background-subtracted DNA signal averaged over the entire vesicle populations, error bars indicate standard deviations. (b) Data points represent individual vesicles. The osmotic difference is the difference in osmolarity between the GUV exterior and interior.

4.4 DNA-mediated vesicle binding

After DNA incorporation experiments, we continued to study the binding of LUVs to GUVs with a DNA-mediated approach. For this reason, we incorporated one chol-DNA2-x in our GUVs, and x-DNA1-chol in our LUVs. We hypothesised that the LUVs are able to bind to a GUV through DNA hybridisation (Figure 4.10a). Contrary to what the schematic figure suggests, individual LUVs cannot be resolved with the epifluorescence microscopy. Instead, a population of bound LUVs appears as a single luminous ring around the GUV membrane (Figures 4.1d-f). As a result, this approach only allowed us to detect LUV localisation on the GUV membrane (i.e. GUV-LUV binding), and did not allow us to distinguish between vesicle docking, hemifusion, and full fusion.

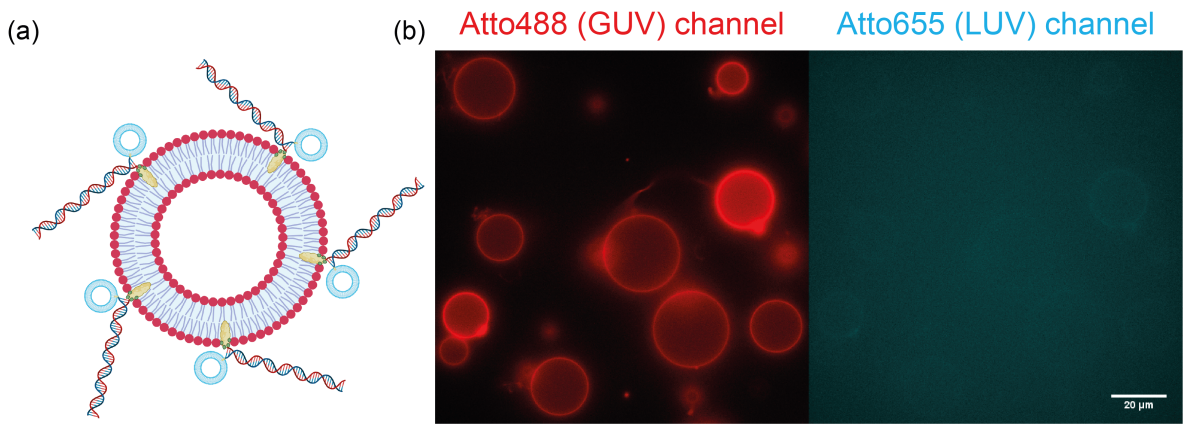


Figure 4.10 (a) Schematic representation of GUV-LUV binding through DNA hybridisation in anti-parallel direction, resulting in a zipper orientation (Figure made with BioRender). (b) Fluorescence crosstalk test of 0.1%Atto488 GUVs without adding LUVs in the LUV channel. Images were made using the GUV-0.1%Atto488 and SUV/LUV-Atto655 imaging settings (Subsection 3.5.3). The scale bar indicates 20 µm.

For GUV-LUV binding, we studied the same parameters as we did for DNA incorporation experiments. For these experiments, GUVs were labelled with 0.1-0.5 mol% Atto488 dye, and LUVs were labelled with 0.05 mol% Atto655. We tested for fluorescence crosstalk by observing GUVs without adding LUVs (Figure 4.10b), and found minimal crosstalk with the imaging settings used (Subsection 3.5.3).

4.4.1 Vesicle binding time

We investigated the timescale of GUV-LUV binding using LUVs extruded through a 200 nm pore, henceforth called 200 nm LUVs. We added the 200 nm LUVs to GUVs and subsequently measured the LUV signal on the GUV membrane at multiple time points (Figure 4.6). Similar to what we observed for DNA incorporation (Figure 4.6), the level of vesicle binding was comparable between the different time points. However, we did find a significant increase in GUV-LUV binding between t = 10 min. and t = 1.5 hr (one-way ANOVA, $p = 0.011$). No significant differences were found between the other samples (one-way ANOVA, $p > 0.05$).

Although we measured a significant difference between the level of vesicle binding from t = 10 min. to t = 90 min., this difference is relatively small, and the samples have relatively large standard deviations (Figure 4.6a). We therefore conclude that a vesicle incubation time of 10 minutes is sufficient to obtain a GUV-LUV binding level close to its maximum.

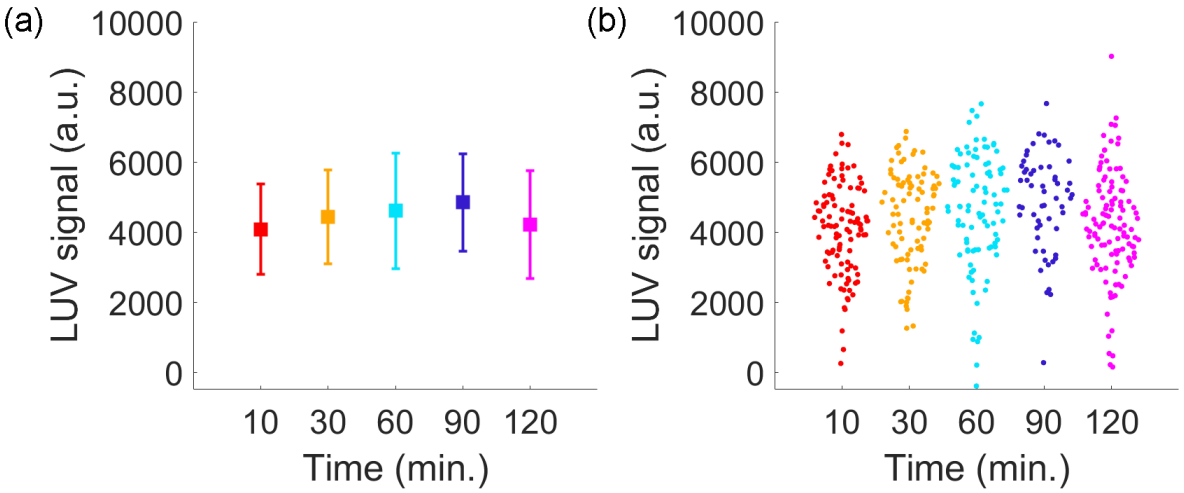


Figure 4.11 Binding of 200 nm LUVs to GUVs for the following time points: t = 10 min. (n = 104), t = 30 min. (n = 91), t = 60 min. (n = 94), t = 90 min. (n = 57) and t = 120 min. (n = 115) using 1 µM DNA. (a) Markers indicate mean level of GUV-LUV binding averaged over entire vesicle populations, and error bars indicate standard deviations. (b) Data points indicate individual vesicles. The LUV signal measured on the GUV membrane is comparable for all time points.

4.4.2 DNA concentration

We investigated the influence of DNA concentration on GUV-LUV binding (Figure 4.12). For each DNA concentration tested, GUVs and LUVs had equal DNA concentrations. We found no significant difference in LUV signal between the samples containing 0 or 0.25 µM DNA (one-way ANOVA, $p = 0.064$). For higher DNA concentrations, the level of GUV-LUV binding increased up to a concentration of 1 µM. Unlike the dependence of DNA incorporation on DNA concentration (Figure 4.8), the level of GUV-LUV binding decreased for concentrations above 1 µM. In fact, we found no significant difference in LUV signal between 0.5 and 5 µM DNA samples (one-way ANOVA, $p = 0.084$). For the remaining samples, the difference in GUV-LUV binding was significant (one-way ANOVA, $p < 0.05$).

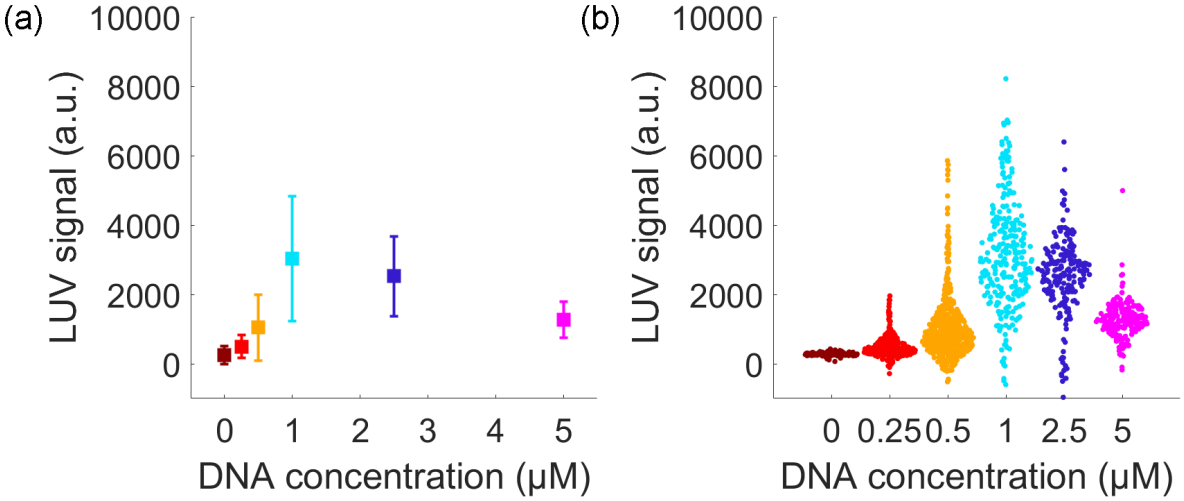


Figure 4.12 Binding of 200 nm LUVs to GUVs for the following DNA concentrations: 0 µM (n = 218), 0.25 µM (n = 307), 0.5 µM (n = 534), 1 µM (n = 271), 2.5 µM (n = 210), and 5 µM (n = 197) µM. Background-subtracted LUV signal represents the LUV signal on the GUV membrane minus the local LUV background signal. (a) Markers indicate mean background-subtracted LUV signals on the GUV membrane averaged over the entire vesicle populations, and error bars indicate standard deviations. (b) Data points indicate individual GUVs.

The decreasing trend for higher DNA concentrations can be explained by three reasons. Firstly, unincorporated ssDNA could bind to ssDNA incorporated in vesicles, thereby blocking vesicle binding. This blockage of ssDNA would especially become apparent for higher DNA concentrations, since the level of DNA incorporation saturates within this range (Figure 4.8a), thereby increasing the number of unincorporated ssDNA (Figure 4.13a). Secondly, the chance that ssDNA partly self-hybridises increases for higher DNA concentrations, thereby preventing LUVs to bind (Figure 4.13b). Self-dimerisation of both ssDNAs used was possible according to the OligoAnalyzer developed by Integrated DNA Technologies [97]. Thirdly, steric hindrance of ssDNA could prevent the binding of LUVs [67] (Figure 4.13c). This would require a high DNA density on the membrane, thereby blocking GUV-LUV binding. Note that ssDNA blockage, self-dimerisation and steric hindrance could occur on the LUV membrane as well, next to the depiction of the processes on the GUV membrane in Figure 4.13.

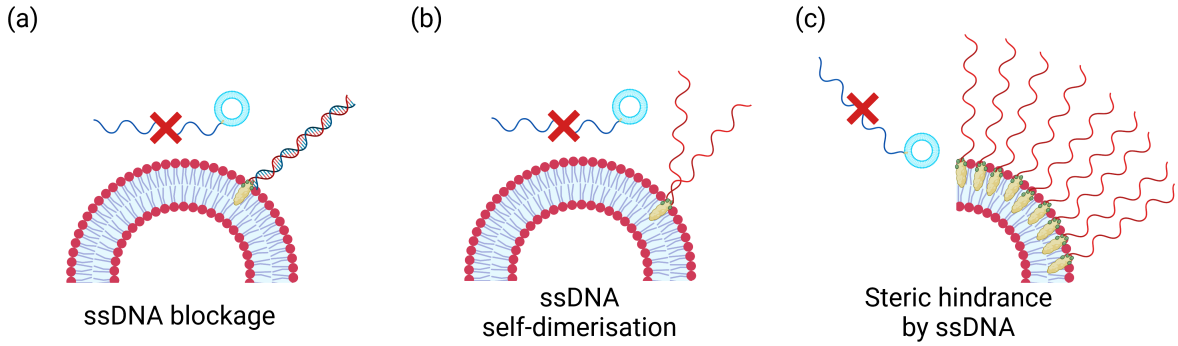


Figure 4.13 Hypotheses explaining a lower level of GUV-LUV binding for higher DNA concentrations. (a) The blockage of ssDNA by unincorporated ssDNA preventing LUV binding. (b) Partial self-dimerisation of ssDNA preventing LUVs to bind. (c) Steric hindrance by ssDNA preventing more GUV-LUV binding. Not that all processes are also possible on the LUV membrane. Figure made with BioRender.

Next to a peak in GUV-LUV binding at 1 µM DNA, this concentration also shows the largest sample variety (Figure 4.12b). This effect could be explained if LUV concentration would be a limiting factor. The number of places where LUVs can bind to GUVs increases with DNA concentration. Consequently, a lack of LUVs would become more apparent at higher DNA concentrations. In this case, not all GUVs could become saturated with LUVs, since the number of places to bind to GUVs is larger than the number of available LUVs. This hypothesis could be tested by increasing the LUV concentration, where we would expect a decrease in sample heterogeneity, and an increase in mean level of binding. The decrease in spread for DNA concentrations higher than 1 µM could be explained by the same hypotheses provided in Figure 4.13, where unincorporated ssDNA, ssDNA self-dimerisation, or steric hindrance decreases the maximum saturation level for binding.

4.4.3 Osmotic conditions

To investigate if increased GUV membrane tension results in more GUV-LUV binding, we varied osmotic conditions in a vesicle binding experiment (Figure 4.14), where comparable osmotic conditions as the DNA incorporation experiment were studied. For the isotonic and hypertonic conditions, significantly more vesicle binding was measured compared to the other osmotic conditions (one-way ANOVA, $p < 0.05$). We would not have expected this based on the DNA incorporation results, since no significant effect of osmotic difference was found there (Figure 4.9). Possibly, membrane fluctuations of deflated GUVs increased the maximum contact area between LUVs docked to GUVs, thereby increasing the chance to form a hemifusion stalk. However, we are uncertain if this would lead to an increased LUV signal on the GUV membrane.

There was no significant difference in GUV-LUV binding between the isotonic and hypertonic samples, nor between the other three samples (one-way ANOVA, $p > 0.05$). We aimed to achieve an osmotic difference of 0 mOsm between the vesicle interior and exterior for the isotonic sample. Since the osmolarity of the buffer used to prepare the isotonic sample deviated from its theoretical value (Subsection 3.8.3), in reality this sample was slightly hypertonic ($\Delta_{Osm} = 8$). An increased LUV signal was no

longer observed for the superhypertonic sample, indicating that the osmotic difference should not be too extreme to optimise vesicle binding. The average level of binding decreased from the isotonic to the superhypotonic sample, suggesting that increasing membrane tension did not increase vesicle binding.

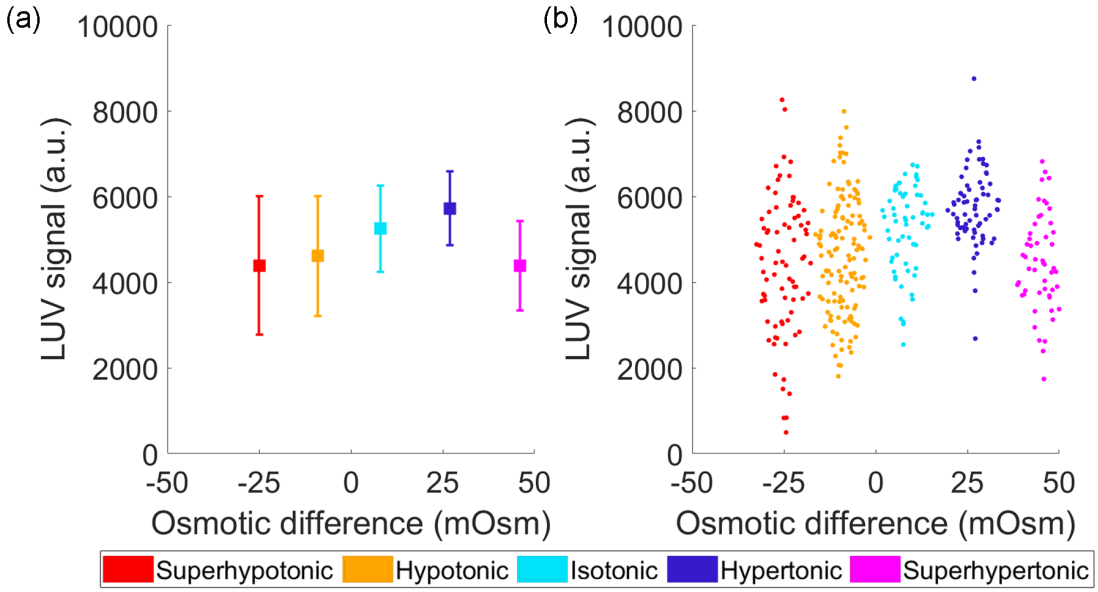


Figure 4.14 The effect of varying osmotic conditions on GUV-LUV binding. Background-subtracted LUV signal for superhypotonic (n = 78), hypotonic (n = 133), isotonic (n = 61), hypertonic (n = 70), and superhypertonic (n = 62) conditions. (a) Markers indicate mean background-subtracted LUV signal averaged over the entire vesicle populations, error bars indicate standard deviations. (b) Data points indicate individual vesicles.

4.5 Bulk fluorescence measurements to develop a content mixing assay

Since the vesicle binding experiments were based on the measurement of LUV lipid dye signal on the GUV membrane, which happened both in the case of vesicle binding and fusion, these experiments did not allow us to visualise fusion. We therefore adopted a content mixing assay to detect fusion as described in Section 2.11. Here, we chose a content mixing assay over a lipid mixing assay, since we were only interested in detecting full fusion, and not in any intermediate fusion states. Using a fluorometer, we investigated the fluorescent behaviour of two dyes to find a suitable candidate for the content mixing assay: sulforhodamine B and HPTS. In literature, fluorescence measurements as well as content mixing experiments of both dyes are described (e.g. [86, 98] for sulforhodamine B, and [99, 100] for HPTS).

In our content mixing assay, we encapsulated the fluorescent dye in our LUVs. Consequently, the fluorescent dye was only visible in GUVs if they underwent fusion. By encapsulating the dye in our LUVs, it also ended up in the outer solution of our final vesicle mixture, albeit diluted. To be able to distinguish fluorescent GUVs from fluorescent signal in the outer solution, we had to quench the fluorescent dye in the outer solution. We therefore tested the possibility to quench the fluorescent dyes as well. Since sulforhodamine B is a self-quenching dye [86], i.e. its fluorescence is reduced at high dye concentrations, there was no need to add a quenching molecule to this dye. Self-quenching capabilites of HPTS were not known, so we adopted a concentration of 7.5 mM of the quencher DPX from Ronan et al. [99] and added this to the HPTS samples.

The results of the fluorescence measurements can be found in Figure 4.15. Here, we can appreciate that the fluorescence of sulforhodamine B increased for 0.01 to 1 mM, but self-quenched at a concentration of 10 mM (Figure 4.15a). For HPTS, a similar trend was observed: an increase of intensity from 0.01 to 10 mM, and a decrease at 100 mM (Figure 4.15b). DPX efficiently quenched HPTS up to a concentration of 25 mM HPTS. The higher fluorescence measured for 25 mM HPTS in presence of DPX is likely due to a shortage of DPX to effectively quench the fluorophore (Figure 4.15c). Indeed, Kreye et al. recommended a DPX concentration 1.5-2 times higher than the HPTS concentration [100]. The HPTS measurements were repeated once with fresh samples, that were measured once more after two days. Here, we found that the HPTS fluorescence measurement was quite replicable and stable for this period of time, especially in the presence of DPX. Since HPTS could more efficiently be quenched than sulforhodamine B, we selected HPTS and DPX as fluorophore-quencher couple for our content mixing assay.

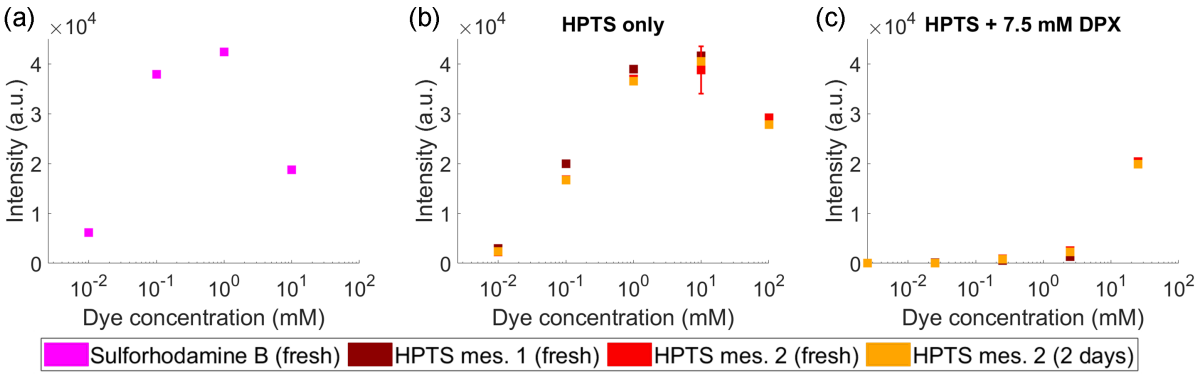


Figure 4.15 Fluorescence measurements of sulforhodamine B (a) and HPTS in absence (b) and presence (c) of its quencher DPX. Fresh HPTS measurements were performed twice (except for 100 mM), and one set of samples was measured again after two days. All individual samples were measured once, except for the measurement (mes.) 2 of the fresh HPTS samples; these samples were split in three and measured twice (n = 6). Markers indicate averages, and error bars are displayed for samples that were measured multiple times. Due to the relatively low standard deviation, the error bars are generally not visible.

4.6 HPTS-DPX content mixing assay

A schematic representation of the content mixing assay can be found in Figure 4.16, where HPTS is encapsulated in LUVs and GUVs only show internal HPTS upon fusion. An example of an experimental observation of the content mixing assay is displayed in Figure 4.17. Here, fused vesicles show a higher internal signal compared to the outer solution in the HPTS channel, while unfused vesicles appear as dark spots. In our experiments, we typically used equal HPTS and DPX concentrations after mixing all vesicle components. However, we later found that it is recommended to quench HPTS with 1.5-2 times as much DPX (Section 3.9. We therefore recommend to increase the DPX concentration to 1.5-2 times the HPTS concentration in future experiments, possibly further decreasing the background signal.

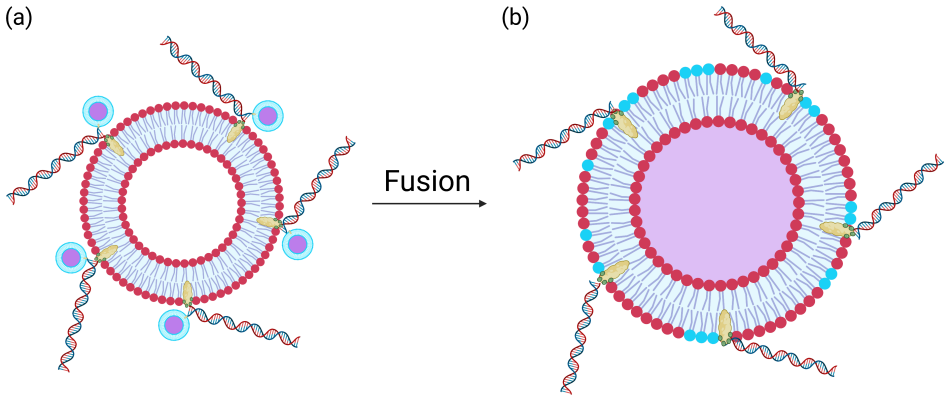


Figure 4.16 Schematic representation of the HPTS-DPX content mixing assay. (a) LUVs are encapsulated with HPTS and bind to the GUV through DNA hybridisation. (b) HPTS is diluted in the GUV interior after successful fusion, and LUV lipids (cyan) are incorporated in the GUV membrane. Figure made with BioRender.

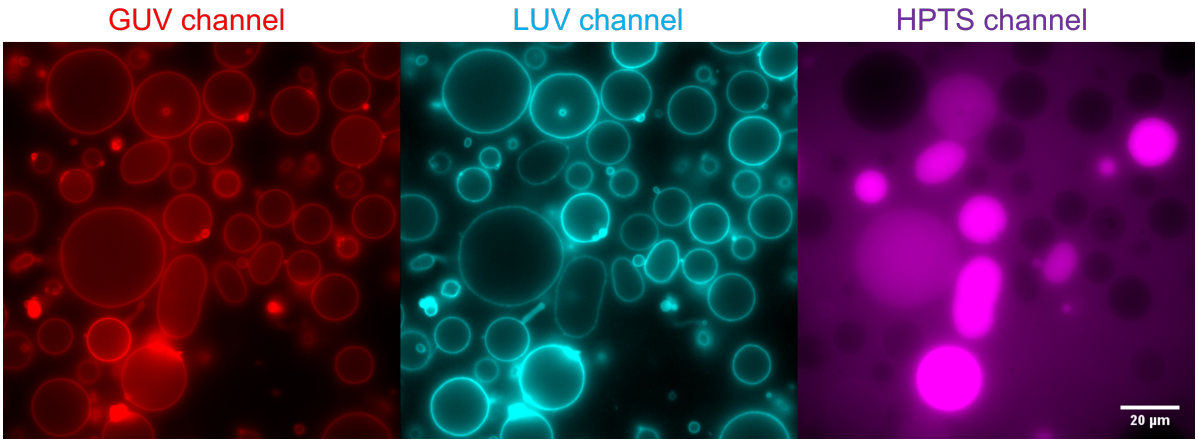


Figure 4.17 Experimental example of the content mixing assay. In the HPTS channel, fused GUVs show an internal signal higher than the outer solution through the delivery of HPTS by LUVs. 0.5% Atto488 GUVs and 0.05% Atto655 200 nm LUVs were used with 1 µM DNA. LUVs were encapsulated with 10 mM HPTS, outer HPTS and DPX concentrations are 2.5 mM. Scale bar indicates 20 µm.

To quantify fusion efficiency, we introduce the **HPTS ratio**, which is the HPTS signal within a GUV divided by the HPTS signal just outside of the vesicle (see Section 3.12 for details on HPTS signal extraction). To distinguish fused vesicles from porous GUVs, where the latter generally had an HPTS intensity similar to the outer solution, we used a cut-off value of 1.05 for the HPTS ratio. Here, we assumed that vesicles with an HPTS ratio above this cut-off value genuinely fused. In this way, we were able to determine the **fusion rate**, i.e. the fraction of GUVs that fused within a vesicle population.

4.7 DNA-mediated vesicle fusion

4.7.1 DNA concentration

Since DNA concentration had such a drastic effect on GUV-LUV binding (Subsection 4.4.2), we first investigated the influence of this parameter on vesicle fusion. Fusion rates were determined by counting the fraction of GUVs with an HPTS ratio above the cut-off value of 1.05, and showed a striking dependence on DNA concentration (Figure 4.18). From 0.25 to 1 µM DNA, the fusion rate increased from 6% to 17%. The most dramatic increase in fusion rate was found from 0.5 to 1 µM DNA, and a similarly dramatic decrease was found from a DNA concentration of 1 to 2.5 µM. For 5 µM DNA, the fusion rate decreased further to 5%, thereby showing a result comparable to 0.25 µM DNA. When no DNA was added, we still found a fusion rate of 3%. This could be caused both by tension-mediated fusion and GUV porosity during GUV-LUV incubation, and will be further discussed in Subsection 4.7.2.

The results displayed here partly correspond with the trend we observed for DNA-mediated vesicle binding (Figure 4.12). For both experiments, the level of binding or fusion peaked at 1 µM DNA. However, we see a difference in how fast the level of binding or fusion decayed. For vesicle binding, the LUV signal of 2.5 µM DNA was comparable to the 1 µM sample, whereas for vesicle fusion, the fusion rate of the 1 µM sample was more than twice as high as the 2.5 µM sample. Since the results for these two experiments were obtained from the same samples, this suggests that fusion is limited faster by an increased DNA concentration than binding. We hypothesise that LUVs could still properly bind to GUVs at a DNA concentration of 2.5 µM, but that they could not be brought sufficiently close to the GUV membrane for fusion, likely due to steric hindrance.

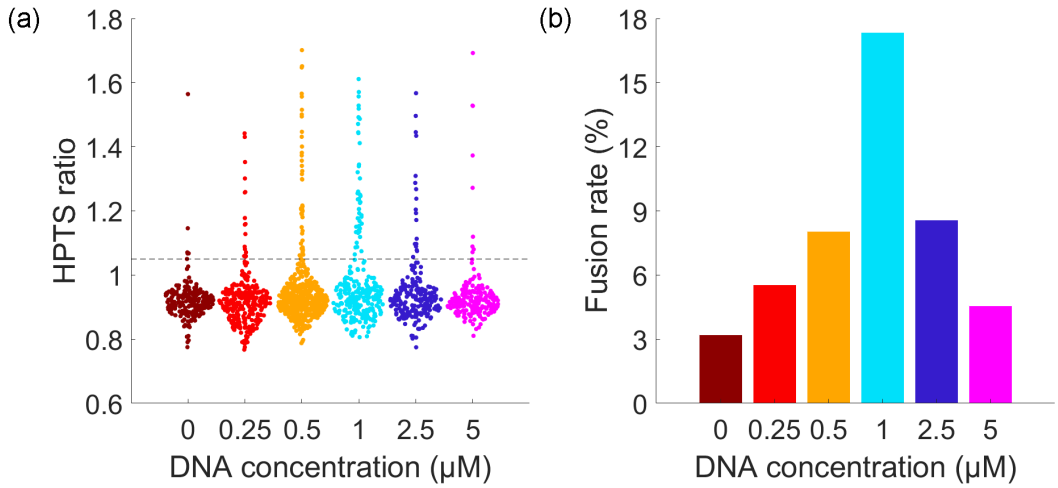


Figure 4.18 The effect of DNA concentration on HPTS ratio (GUV internal HPTS signal divided by outer HPTS signal) and fusion rate (fraction of fused vesicles). (a) Swarm plot for 0 µM (n = 218), 0.25 µM (n = 307), 0.5 µM (n = 534), 1 µM (n = 271), 2.5 µM (n = 210), and 5 µM (n = 197)DNA. Each data point indicates an individual vesicle, and the cut-off value for fusion of 1.05 is indicated with the dashed line. (b) Fusion rates for the different DNA concentrations.

The fact that both GUV-LUV binding and fusion peaked at 1 µM suggests that fusion was enhanced for higher levels of GUV-LUV binding. To test this hypothesis, we assessed the correlation between the LUV signal and HPTS ratio over the entire vesicle population of this experiment, where the no DNA control sample was excluded (Figure 4.19a). Here, we found a small but significant correlation between the two parameters (Pearson correlation test, $r = 0.11$, $p = 1.0 \cdot 10^{-5}$). However, we should be careful in interpreting this data, since HPTS ratio negatively correlates with GUV size (Figure 4.19b). This negative correlation was caused by the large contribution of out-of-focus fluorescence in epifluorescence microscopy, giving larger unfused vesicles a lower internal HPTS signal than smaller unfused vesicles. We found no significant correlation between GUV size and LUV signal (4.19c). Here, it should be noted that the HPTS signal was affected more by out-of-focus signal than the LUV signal, since HPTS generally showed a higher background signal.

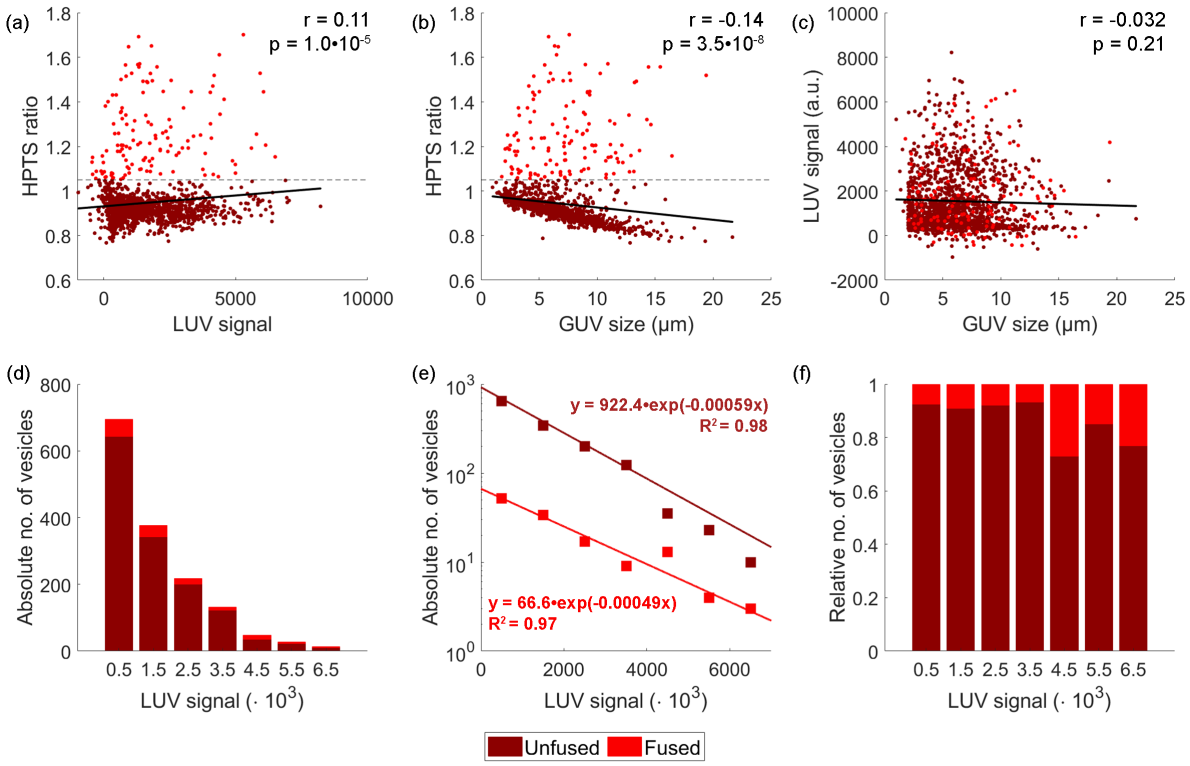


Figure 4.19 Analysis over the entire population of vesicles with DNA (n = 1519). The markers in the scatter plots (a-c) indicate individual vesicles, the black lines represent linear fits, and r and p the Pearson correlation coefficients and p-values, respectively. A significant correlation between background-subtracted LUV signal and HPTS ratio is found (a), but this could be influenced by the negative correlation between GUV size and HPTS ratio (b). No significant correlation is found between GUV size and background-subtracted LUV signal (c). The total number of vesicles decays exponentially with LUV signal (d, bar width = $1{\cdot}10^3$), where the exponential decay is stronger for unfused vesicles (e). Exponentials were fitted by taking the middle value of each bar in (d), and by assigning the number of fused or unfused vesicles to these values. The fraction of fused vesicles is higher for LUV signals above $4.0{\cdot}10^3$ (f). Two outliers with LUV signal above $7.0{\cdot}10^3$ were discarded for the analysis.

Interpretation of the data becomes easier when converting the continuous HPTS ratio to a binary classification of fused and unfused vesicles. In Figure 4.19d, it is displayed how the absolute number of vesicles decays exponentially with measured LUV signal. We found exponential fits for $y_{unfused} = 9.2{\cdot}10^2{\cdot}\exp(-5.9{\cdot}10^{-4}{\cdot}x)$ ($R^2 = 0.98$) and $y_{fused} = 67{\cdot}\exp(-4.9{\cdot}10^{-4}{\cdot}x)$ ($R^2 = 0.97$), showing that the exponential decay of fused vesicles is somewhat less strong than it is for unfused vesicles (Figure 4.19e). Corresponding to this, the fractions of fused and unfused vesicles reveal that the relative amount of fused vesicles was higher for LUV signals above $4.0{\cdot}10^3$ (Figure 4.19f). In Subsection 4.4.2, it was shown that an LUV signal above $4{\cdot}10^3$ was especially common for vesicles containing 1 µM DNA (Figure 4.12b), the concentration for which we found the highest fusion rate. Together, these results suggest that fusion was enhanced at higher levels of LUV binding. To strengthen this statement, we would require high-resolution microscopy techniques, to be able to better quantify LUV signal on the GUV membrane, and to eliminate the GUV size dependence on measured HPTS ratio.

4.7.2 Osmotic shock

In fusion experiments, GUVs were incubated with LUVs for 60-80 minutes before they were added to observation buffer to visualise them (Section 3.10). Since the LUV buffer had a lower osmolarity than the GUV buffer (260 vs. 320 mOsm), this put the GUVs temporarily under an osmotic shock of ~-30 mOsm. As described in Section 2.7, it has been shown that increasing membrane tension enhances the fusion rate [30]. With our fusion method, we therefore combined the DNA and tension-based approaches with the goal to further increase fusion rate.

Next to increasing the fusion rate, putting GUVs under tension possibly made them more porous, increasing the chance of HPTS leaking from the outer solution into the vesicles (Section 4.6). Since the quencher DPX was added only after the fusion step, transient pore formation of GUVs could result in an internal HPTS signal that is higher than the outer solution. To investigate if we observed these false positives for fusion, and to find out to what extent the osmotic shock contributes to this, we performed the fusion protocol in presence and absence of the osmotic shock. The osmotic shock was eliminated by approximately matching the osmolarity of the GUVs and LUVs. Next to the effect of the osmotic shock, the influence of DNA on membrane porosity was investigated.

In Figure 4.20, the level of GUV-LUV binding (a), HPTS ratios (b) and fusion rates (c) for the different samples are displayed. The samples with DNA on both GUVs and LUVs clearly show higher values for the LUV signal, HPTS ratio, and fusion rate compared to the other samples. These results showcase that our protocol allows for specific fusion, where the majority of GUVs and LUVs only fuse when they both contain DNA. In (c), we can possibly see some effect of membrane porosity. For the samples without DNA or only DNA on the GUVs, we would not expect DNA-mediated fusion. However, higher fusion rates were detected for the samples with osmotic shock (3% and 5%) compared to the samples lacking an osmotic shock (1% for both samples). This suggests that vesicles in these samples were either more porous, or showed an enhanced fusion rate due to tension-mediated fusion.

Although we observed an increased LUV signal on some of the vesicles without DNA, there was no significant difference between the level of GUV-LUV binding for the first four samples (one-way ANOVA, $p > 0.05$). There neither was a significant difference in LUV signal for the latter two samples (one-way ANOVA, $p = 0.62$). This corresponds with the experiment where we investigated the influence of osmotic conditions on GUV-LUV binding, where we found little effect of this parameter (Subsection 4.4.3). Interestingly, the fusion rate of the vesicles with DNA on GUVs and LUVs increased by 74% for the sample with osmotic shock compared to the sample without osmotic shock. Together, these results show that increasing membrane tension had little effect on vesicle binding, but greatly enhanced fusion rate. Although the increase in fusion rate may be partly attributed to membrane porosity, the absolute difference in fusion rate for the latter two samples is still larger than we observed for the samples lacking DNA.

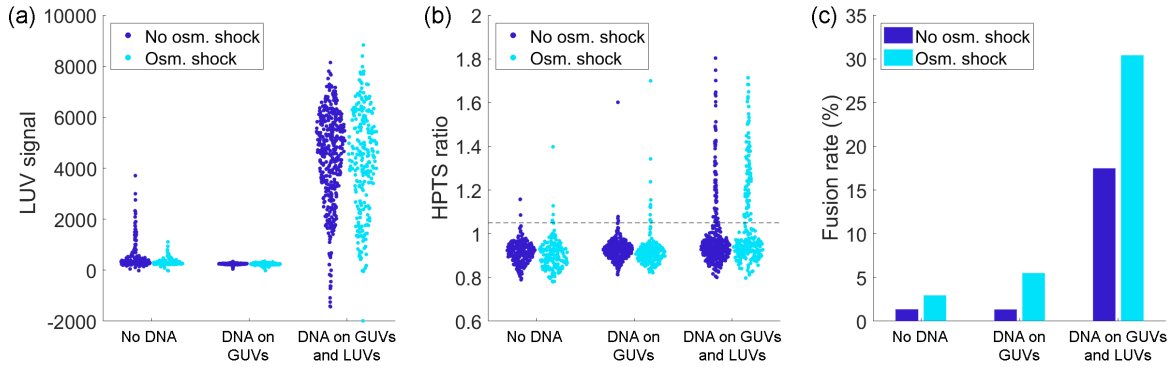


Figure 4.20 The effect of an osmotic shock on fusion efficiency and vesicle porosity. Background-subtracted LUV signals on the GUV membrane (a), HPTS ratios (b) and fusion rates (c) for vesicles without DNA (n = 225 for no osm. shock and n = 169 for osm. shock), with DNA on GUVs (n = 295 for no osm. shock and n = 235 for osm. shock) and with DNA on GUVs and LUVs (n = 411 for no osm. shock and n = 250 for osm. shock) with or without osmotic (osm.) shock. Data points in (a) and (b) represent individual vesicles, and (c) the fusion rates of the whole population samples.

4.7.3 LUV size

Since it was found that smaller vesicles are more fusogenic (Section 2.6) [42, 43], we investigated the influence of LUV size on fusion. Although an increased fusogenicity of smaller vesicles could be beneficial to obtain more membrane growth, it also means that more fusion event are required to significantly increase the surface are of a GUV. The surface area of a vesicle scales quadratically with its radius. Therefore, four 100 nm LUVs are required to deliver the same amount of membrane area

as one 200 nm LUV. We therefore have to find a balance between increased fusogenicity of smaller vesicles, and the larger amount of membrane area delivered to a GUV per fusion event.

To test how LUV size affects vesicle fusion, we incubated GUVs with either 100 or 200 nm LUVs and compared fused with unfused vesicles (Figure 4.21). Here, we found comparable fusion rates for both LUV sizes ((Figure 4.21b, 25% for 100 nm LUVs and 23% for 200 nm LUVs). Due to their difference in volume, 100 nm LUVs have to fuse eight times as often as 200 nm LUVs to deliver the same amount of HPTS to a GUV. The comparable fusion rates for the two LUV types therefore suggests that 100 nm LUVs are considerably more fusogenic than 200 nm LUVs, corresponding to earlier work. Since we do not know how many fusion events are required to detect fusion, we are uncertain about the extent of increased fusogenicity for 100 nm LUVs.

Interestingly, we only found a significantly larger level of GUV-LUV binding for fused vesicles compared to unfused vesicles for the 100 nm sample (Figure 4.21c, one-way ANOVA, $p = 0.046$). For the 200 nm LUV sample, no significant difference was found between GUV-LUV binding of fused and unfused vesicles (one-way ANOVA, $p = 0.95$). Figure 4.21c also shows that more 100 nm LUVs were bound to GUVs than 200 nm LUVs, corresponding to their increased fusogenicity. In Figure 4.21d, the sizes of fused and unfused vesicles are compared. Strikingly, no significant difference in GUV size was found for the 100 nm LUV samples (one-way anova, $p = 0.99$), while a significant increase in GUV radius of 41% was measured for 200 nm LUVs (one-way ANOVA, $p = 3.77 \cdot 10^{-9}$, $\langle r_{unfused} \rangle = 8.0$ µm, $\langle r_{fused} \rangle = 11.3$ µm). Since there was no significant difference in GUV size between the first three samples (one-way ANOVA, $p > 0.05$), it is likely that the increase in radius for GUVs fused with 200 nm LUVs was caused by membrane growth.

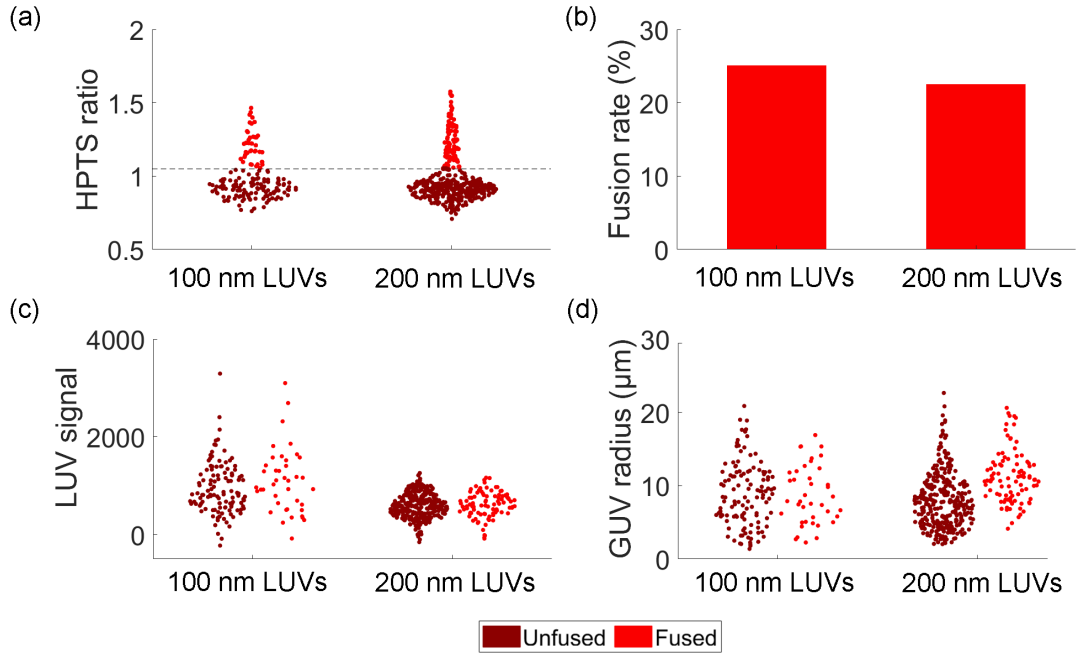


Figure 4.21 The effect of LUV size on GUV-LUV fusion. We compared unfused GUVs with GUVs fused with 100 (n = 163) or 200 nm (n = 416) LUVs. We measured HPTS ratio (a), fusion rate (b), background-subtracted LUV signal on the GUV membrane (c), and GUV radius (d). Data points of the swarm plots indicate individual vesicles.

We hypothesise that the smaller size of 100 nm LUVs compared to 200 nm LUVs resulted in no measured increase in average GUV radius for the 100 nm sample. This suggests that, in our case, the larger size of 200 nm LUVs is more advantageous to obtain membrane growth compared to the increased fusogenicity of 100 nm LUVs. However, it should be noted that the sample size of GUVs fused with 100 nm LUVs is relatively small, and that an increase in average GUV radius could potentially be detected when using a larger sample size.

4.7.4 GUV lipid composition and formation method

The majority of fusion experiments were performed with GUVs and LUVs consisting for the largest part out of the lipid DOPC, with a small fraction of 0-0.5% lipid dye. In the context of a synthetic cell, it would be valuable if our fusion protocol also works for lipid compositions that more closely resemble real cells. Eukaryotic cells typically contain a substantial fraction of negatively charged lipids and cholesterol in the form of lipid rafts [101]. We therefore tested the capability of physiologically relevant GUVs to fuse by including 20% negatively charged DOPS lipids and 20% cholesterol next to DOPC and lipid dye, corresponding to lipid compositions observed in living cells [55].

Lipids containing a PE head group have been found to promote the formation of the hemifusion stalk [39], and the formation of a fusion pore in some cases [40] (Section 2.6). We therefore tested the fusogenicity of vesicles containing 30% POPE next to DOPC and lipid dye. Finally, we also investigated the effect of GUV formation method on fusion. We did this by including vesicles formed by eDICE instead of gel swelling. The eDICE vesicles consisted out of DOPC and lipid dye. For all samples, controls where only GUVs contained DNA were included.

The effect of lipid composition and GUV formation method on GUV-LUV binding and fusion is displayed in Figure 4.22. eDICE vesicles had a low GUV signal, and no GUV-LUV binding was observed for the eDICE control (Figure 4.23). As a result, we were not able to properly detect vesicles in this sample. This sample was therefore not included in the analysis. In Figure 4.22, we can appreciate that there was little to no GUV-LUV binding for the control samples, whereas all other samples showed an increased LUV signal. Next to this, fusion was achieved for the different lipid compositions and fusion methods, showcasing the broader applicability of the fusion protocol.

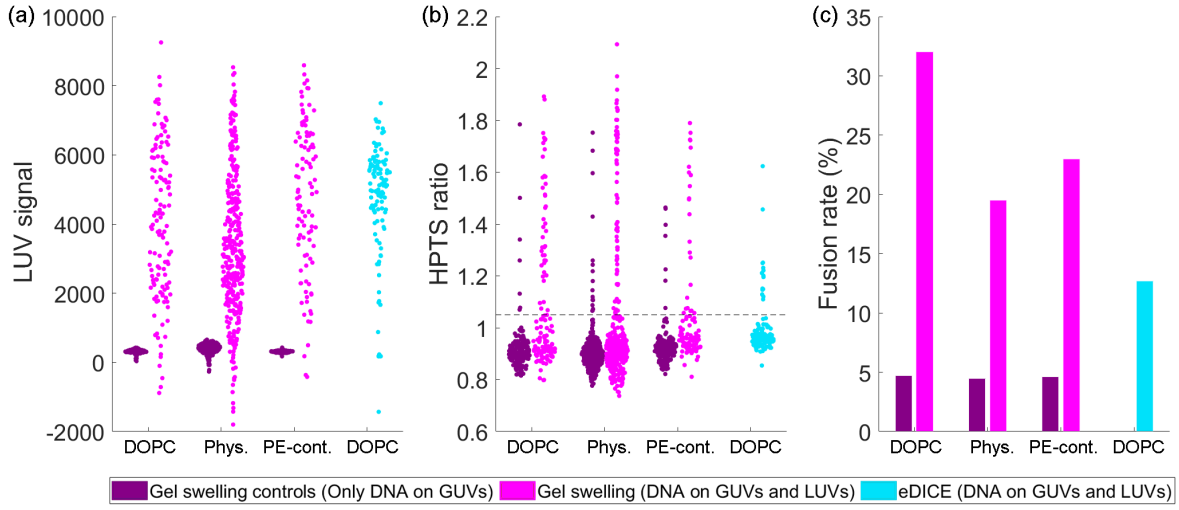


Figure 4.22 Effect of GUV lipid composition and GUV formation method on fusion efficiency. We assessed GUV-LUV binding (a), HPTS ratio (b), and fusion rate (c). Data points of the swarm plots indicate individual vesicles. For gel swelling, DOPC GUVs (n = 149 for control, n = 129 for no DNA control), physiologically relevant GUVs (phys., n = 379 for control, n = 369 for no DNA control), PE-containing GUVs (n = 152 for control, n = 100 for no DNA control) were used. Next to this, DOPC GUVs formed by eDICE were used (n = 118).

The highest fusion rate was detected for DOPC vesicles formed by gel swelling. However, this could be due to the optimisation of the fusion protocol for this vesicle type in terms of DNA concentration and osmotic conditions. Interestingly, despite its lower fusion rate, the PE-containing sample showed significantly more GUV-LUV binding than the DOPC sample formed by gel swelling (one-way ANOVA, $p = 0.0062$). This corresponds to the findings of Chernomordik et al., where cone-shaped lipids promoted the formation of a hemifusion stalk, but prevented the formation of a fusion pore [39]. The LUV signal of the physiologically relevant GUVs was lower compared to all other samples (one-way ANOVA, $p > 0.05$, excluding the no DNA controls), which might be due to electric repulsion of the negatively charged DOPS lipids and DNA.

DOPC vesicles formed through eDICE were smaller and showed a lower GUV signal compared to DOPC vesicles formed by gel swelling (Figure 4.23). This possibly indicates that eDICE vesicles did not form optimally. Potentially, the fusion rate of eDICE vesicles could be increased if vesicle formation is more successful.

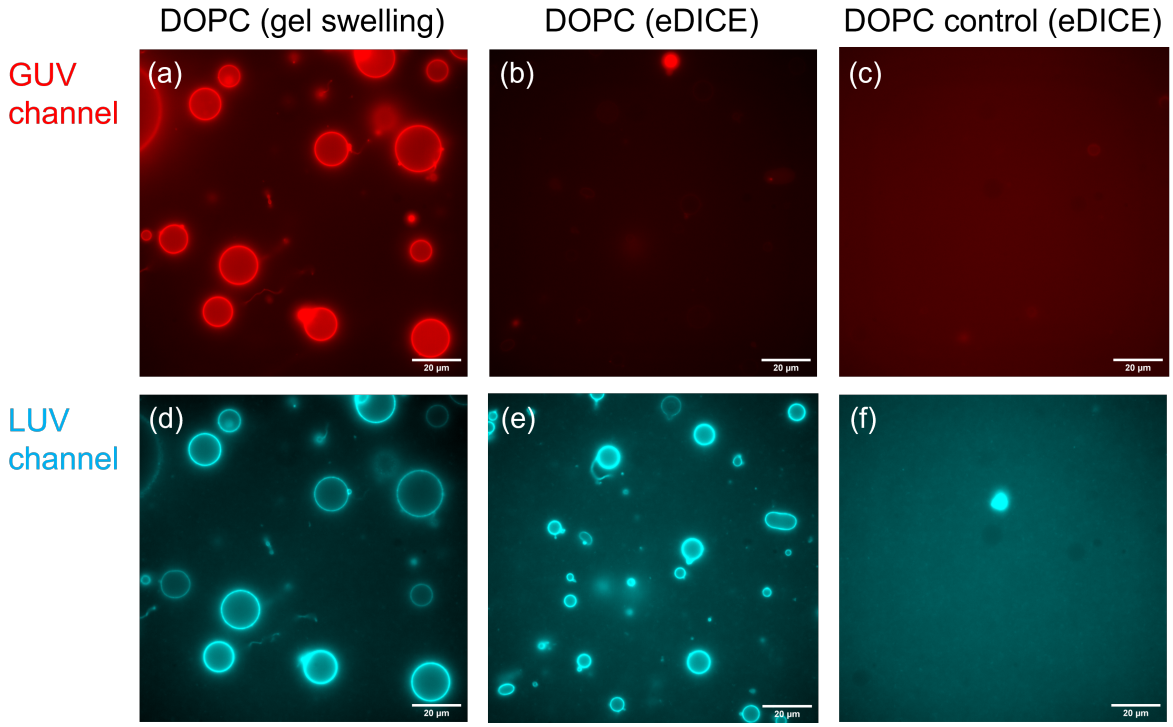


Figure 4.23 Comparison of 99.95% DOPC + 0.5% Atto488 GUVs formed by gel swelling (a) or eDICE (b and c). Atto655 LUVs were added with DNA (a and b) or without DNA (c). eDICE vesicles showed a lower GUV signal, and no LUVs bound to GUVs in the eDICE control. Scale bars indicate 20 µm.

4.7.5 GUV lipid dye

In earlier work on DNA-mediated fusion, it was found that lipid dye could mediate DNA-independent lipid mixing [66]. We therefore investigated the influence of the lipid dyes we used for our GUVs on fusion efficiency. We found a somewhat higher fusion rate for unlabelled GUVs (20%, n = 364) compared to GUVs labelled with 0.5 % Atto488 (16%, n= 545),[4] the lipid dye we typically used to visualise GUVs in fusion experiments. For GUVs labelled with 0.5% Atto655, we found a lower fusion rate of 8% (n = 283), suggesting that usage of this dye decreases fusion efficiency. However, the Atto655 GUV sample had relatively more aberrant structures in the form of small GUVs sticking to each other compared to the Atto488 sample (Figure 4.24). These aberrant structures possibly decreased the fusion rate by incorporating DNA that could no longer be used for fusion.

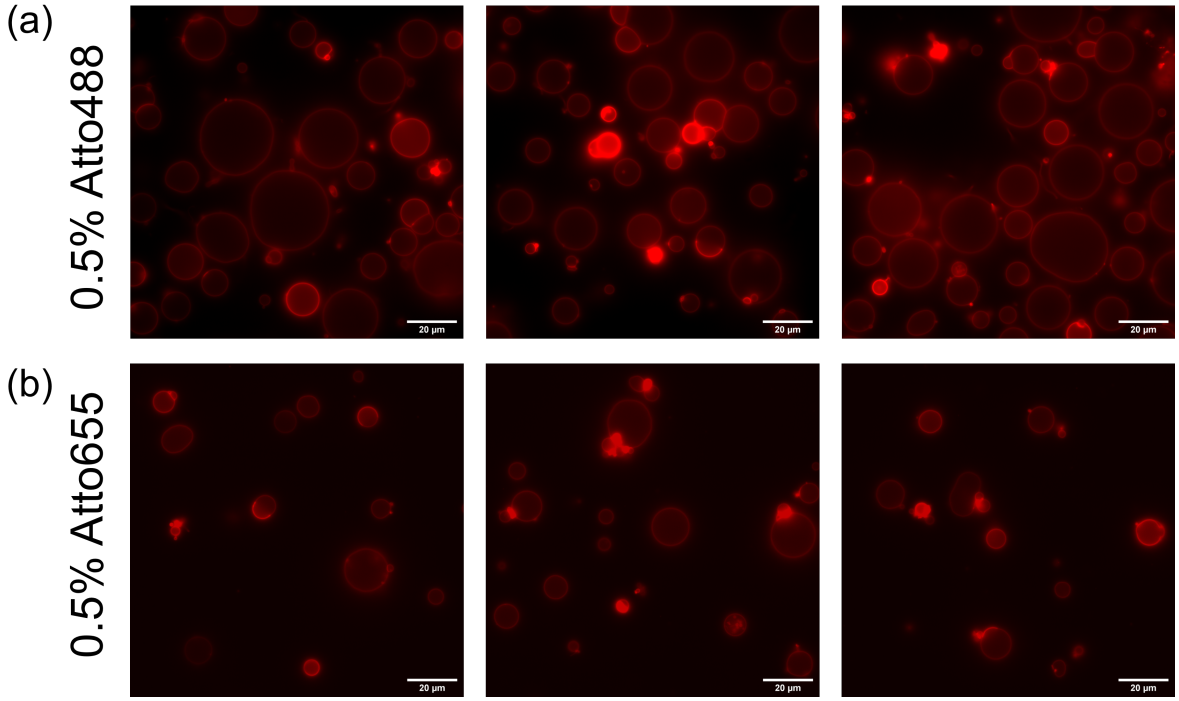


Figure 4.24 The effect of lipid dye on DNA-mediated GUV-LUV fusion. Comparison of 99.95% DOPC + 0.5% Atto488 GUVs (a) and 99.95% DOPC + 0.05% Atto655 GUVs (b). GUVs were incubated overnight with 1 µM chol-DNA2-x and mixed with 200 nm unlabelled LUVs that were incubated overnight with 1 µM x-DNA1-chol. 0.05% Atto655 GUVs showed relatively more aberrant structures, possibly contributing to its lower fusion rate compared to unlabelled GUVs and 0.5% Atto488 GUVs. Scale bars indicate 20 µm.

4.7.6 Vesicle mixing order and KCl concentration

From experimental observations, we found that the order of mixing vesicles greatly affected fusion efficiency. Specifically, we found a fusion rate of 10% (n = 222) when GUVs and LUVs were first incubated with each other for 60-80 minutes before being added to quenching OBS (i.e. "the working fusion protocol"), compared to a fusion rate of 0% (n = 197) when GUVs and LUVs were directly mixed in quenching OBS (i.e. the "ineffective fusion protocol"). In this specific experiment, we used a higher DNA concentration of 2.5 µM compared to 1 µM for other experiments. This experiment was also done in parallel with an experiment where we tested the effect of KCl concentration on GUV-LUV binding. Consequently, the reported fusion rates were for vesicles in a solution of 10 mM KCl, in contrast to 100 mM KCl for other experiments. This is the only experiment where we directly compared the effect of vesicle order. However, all other experiments showing high fusion rates described in this study were

[4] Since we used no lipid dye for LUVs in this experiment, we were not able to detect the membranes of unlabelled GUVs for analysis. We therefore determined the fusion rates of this experiment by manually counting the number of fused vesicles.

performed with the working fusion protocol. Experiments of the systematic check of the working fusion protocol (Appendix B) were performed with slight adaptions of the old fusion protocol, but resulted in fusion rates of 0%. The disadvantage of the working fusion protocol is that it does not allow for the observation of live fusion, since vesicles are only added to observation buffer once they have already fused. Observing live fusion would allow us to gain a deeper understanding of the fusion process, and to better quantify the amount of membrane growth.

In Figure 4.25, the results of the combined experiment investigating vesicle order and KCl concentration are displayed. The level of GUV-LUV binding for the working fusion protocol was drastically higher than it was for the old fusion protocol, which likely explains the difference in fusion efficiency between the two fusion protocols. We hypothesise that the increased level of GUV-LUV binding and fusion efficiency for the working fusion protocol was caused by the reversible binding of cholesterol-tagged DNA in vesicle membranes. In the discussion, we will elaborate on this hypothesis (Subsection 5.8.3). Before we came up with this hypothesis, we performed a systematic check to assess other possible explanations for the efficacy of the working fusion protocol (Appendix B). Next to a difference in GUV-LUV binding between the two fusion protocols, we observe enhanced binding for the higher KCl concentrations. This is in line with earlier result of Morzy et al., who showed that ~100 mM monovalent cations are required to optimise cholesterol-tagged DNA incorporation [73].

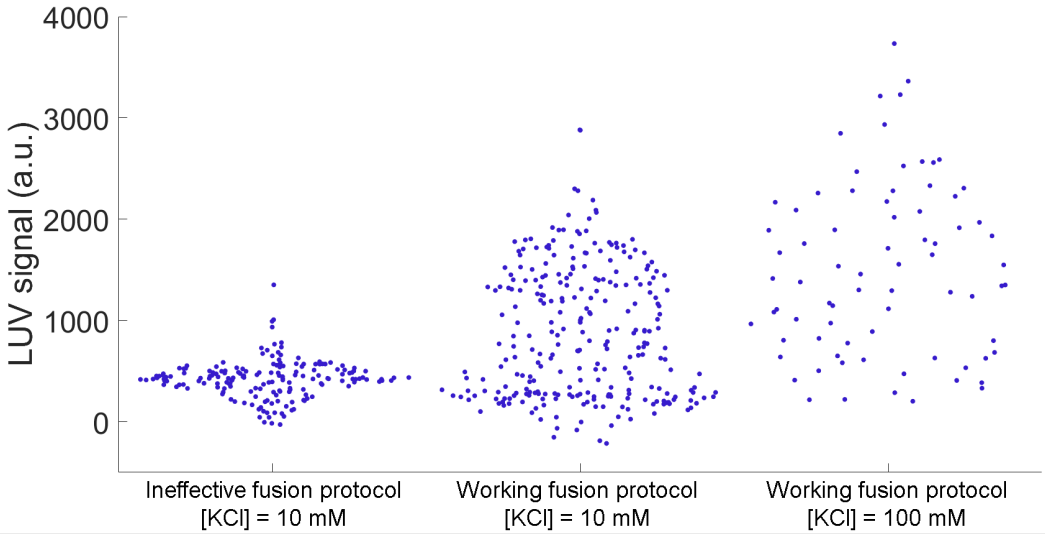


Figure 4.25 The effect of vesicle mixing order and KCl concentration on GUV-LUV binding. Background-subtracted LUV signal on the GUV membrane for the ineffective fusion protocol (n = 174), the working fusion protocol with 10 mM KCl (n = 273) and the working fusion protocol with 100 mM KCl (n = 77). 0.1% Atto488 GUVs were mixed with 200 nm Atto655 LUVs, where both vesicle types were incubated with 2.5 µM DNA. Data points indicate individual vesicles.

Since we found no explanation for the efficacy of the working fusion protocol in time, we could not adapt it in such a way to allow for the observation of live fusion. With the goal to be able to observe live fusion, we tested existing and newly developed fusion detection methods (Appendix C). Next to this, we were able to measure a constant surface area for GUVs trapped in a micropipette aspriation (MPA) set-up (Appendix D), which is required to study membrane growth of individual vesicles in future experiments. Finally, we were able to locally inject LUVs to GUVs in solution, showing rapid LUV localisation on the GUV membrane (Appendix D).

4.8 Indications of membrane growth

In Subsection 4.7.3, we showed that the mean radius of GUVs fused with 200 nm LUVs was 41% higher than for unfused GUVs. We found that this size difference was a common trend among samples of different fusion experiments. In Figure 4.26, the samples where we measured significant differences in mean radius between fused and unfused GUVs are shown (one-way ANOVA, $p < 0.05$). Here, the experiments are indicated below each set of markers, and the specific samples are indicated in brackets. The measured differences in mean GUV radius range from 23% for the 1 µM DNA sample of the DNA concentration experiment to 66% for the PE-containing GUVs of the lipid composition experiment. The differences in mean GUV radius could partly be explained by the swelling of tense GUVs, that are more fusogenic, but this can only increase the surface area of a GUV with ~5% [102]. We therefore hypothesise that the increased size of fused GUVs was for the largest part caused by membrane growth. This hypothesis is supported by the fact that the samples exhibiting a significant GUV size increase typically were the samples with the highest fusion rate(s) of an experiment.

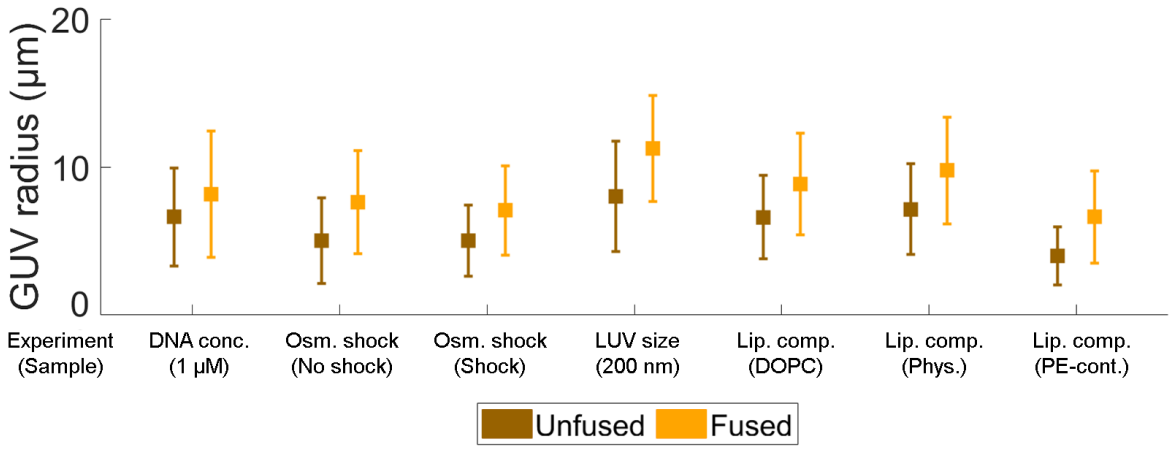


Figure 4.26 Samples where we observed a significant difference in GUV radius between fused and unfused vesicles. The experiment refers to a fusion experiment described in Section 4.7, and the specific samples are indicated in brackets. Markers indicate average radius of the unfused or fused vesicle population, error bars indicate standard deviations.

In Table 4.1, the fusion rates, relative size differences, and relative size differences squared corresponding to the samples in Figure 4.26 are visible. If GUV size differences were indeed for the largest part caused by membrane growth, the relative size differences squared indicate the average amount of membrane growth fused GUVs underwent. This would mean that the surface area of GUVs showed a striking ~1.5-2.8 fold increase. However, future experiments where fusion is observed in real-time are required to confirm that it is indeed membrane growth we observed.

Experiment (Sample, Subsection)	Fusion rate (%)	$\frac{<r_{Fused}>}{<r_{Unfused}>}$	$(\frac{<r_{Fused}>}{<r_{Unfused}>})^2$
DNA concentration (1 µM DNA, Subsection 4.7.1)	17	1.23	1.52
Osmotic shock (No shock, Subsection 4.7.2)	18	1.52	2.31
Osmotic shock (Shock, Subsection 4.7.2)	30	1.41	1.99
LUV size (200 nm LUVs, Subsection 4.7.3)	23	1.41	1.98
Lipid composition (DOPC GUVs, Subsection 4.7.4)	32	1.34	1.79
Lipid composition (Phys. relevant GUVs, Subsection 4.7.4)	20	1.37	1.86
Lipid composition (PE-containing GUVs, Subsection 4.7.4)	23	1.66	2.75

Table 4.1 Fusion rates and GUV size increases for different experiments of the project. The relative size increase squared indicates the average amount of membrane growth achieved for fused vesicles, assuming that GUV size increase is fully caused by membrane growth.

The samples where we found no significant increase in mean GUV radius were the samples where GUVs had DNA concentrations other than 1 µM, were fused with 100 nm LUVs, or were formed by eDICE (one-way ANOVA, $p > 0.05$). We hypothesise that we observed no size difference in these cases since the levels of fusion were lower, or since 100 nm LUVs were not able to sufficiently increase the membrane area of GUVs upon fusion.

Next to the increase in mean GUV radius, fused vesicles were often elongated (Figure 4.27) or fluctuating (Video S2, available online),[5] which could also indicate membrane growth. We also observed elongated or fluctuating vesicles showing no internal HPTS, but it seemed like the occurrence of these phenomena was less common for unfused vesicles. To find out if fused vesicles indeed show this behaviour more often than unfused vesicles, quantification of the shape and degree of fluctuation of vesicles is required.

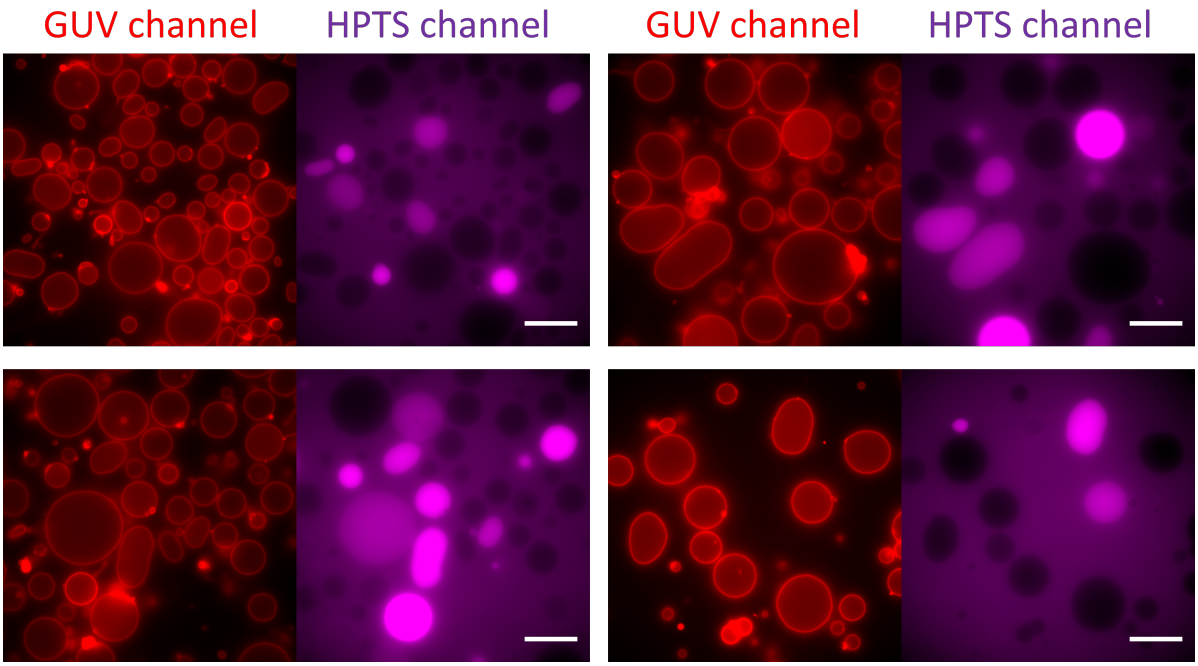


Figure 4.27 Fused vesicles were often elongated, possibly indicating membrane growth. Images were taken from 0.5% Atto488 GUVs and 200 nm Atto655 LUVs with 1 µM DNA. Scale bars indicate 20 µm.

[5] Video of the bottom-left image of Figure 4.27 in the GUV channel. Fluctuating vesicles with internal HPTS are indicated with arrows.

5
Discussion

5.1 DNA and tension-mediated fusion for *in vitro* membrane growth

An increase in membrane area of 26% is required for cells to divide during cytokinesis [17]. Apart from cytokinesis, however, cellular growth occurs throughout the cell cycle [5]. Essentially, in order to keep cellular size constant throughout multiple division and regrowth cycles (i.e. sustained cell division), it should double both in terms of plasma volume and membrane area. Cellular volume can be controlled relatively easily via the transport of water through aquaporins, osmosis, or diffusion [103]. An increase in membrane area, on the other hand, can only be achieved through complex processes involving lipid synthesis or membrane fusion.

In the context of building a synthetic cell, membrane growth should be reconstituted with a bottom-up approach. We aimed to achieve this by fusing GUVs with LUVs. We chose for these specific vesicle types because of their extensive description in literature and their broad experimental applicability [20, 21]. Moreover, a GUV is the chassis chosen to build a synthetic cell in the BaSyC project. With the goal to achieve a substantial amount of membrane growth, we combined fusion approaches based on membrane tension and DNA hybridisation. Membrane tension has recently been shown to promote fusion between GUVs and SUVs [30], but offers no selective fusion mechanism. A fusion strategy based on DNA, on the other hand, does allow for selective fusion through the usage of two complementary DNA strands. Next to this, it is a relatively easy fusion approach that is compatible with other synthetic cell modules.

DNA-mediated fusion has been demonstrated extensively for similarly sized vesicles [23], and more recently for the fusion of GUVs with LUVs [16]. However, the optimal conditions for DNA-mediated GUV-LUV fusion, and the way in which this approach interacts with membrane tension, remains unknown. In this study, we explored the parameter space of DNA and tension-mediated fusion using semi-quantitative epifluorescence imaging, thereby deriving GUV-LUV fusion characteristics on a population level. Here, we achieved fusion for up to ~30% of GUVs under the most optimal conditions. In no DNA control samples, we found a background fusion rate of ~5%, which was either caused by tension-mediated fusion or vesicle leakage. The experiments also gave insights in the process of membrane fusion in general, a mechanism that is relevant to many biological processes such as egg cell fertilisation, cellular signalling and viral infection [79].

We adopted a content mixing assay to detect fusion. Here, we encapsulated the fluorescent dye HPTS in LUVs, that subsequently showed signal from the interior of GUVs upon successful GUV-LUV fusion. As pointed out by Mora et al., detecting fusion between GUVs and LUVs is challenging because of the dramatic difference in size between these vesicle types [82]. In our case, this means that a large number of GUV-LUV fusion events was required before fusion was detectable. We approached this by encapsulating a high concentration of HPTS in LUVs (10 mM), thereby maximising the amount of HPTS delivered to GUVs per fusion event. Next to this, we quenched the outer vesicle solution with DPX to be able to better detect the increased HPTS signal of fused GUVs. Based on the results of our bulk fluorescence measurements, we estimate that the number of fusion events required to detect fusion were in the order of $\sim 10^2$ for 200 nm LUVs and $\sim 10^3$ for 100 nm LUVs.

5.2 DNA incorporation in GUVs saturates at a concentration of 2.5 µM

We investigated the effect of DNA concentration on DNA incorporation, GUV-LUV binding and GUV-LUV fusion. The studies discussed in Chapter 2 generally used irreversible DNA-anchors and could therefore estimate the amount of DNA strands per vesicle through lipid and DNA molar ratios. Earlier work, however, showed that the single cholesterol anchor we used binds reversibly to SLBs [95, 96]. This is in line with our findings, where we observed a relatively narrow distribution for the level of DNA incorporation in GUV membranes. Consequently, we could not exactly calculate DNA:vesicle ratios for our experiments. To still get some insights in the DNA density on the vesicles used in this study, we calculated the maximum DNA:vesicle and DNA:lipid ratios for vesicles used in our experiments (Table 5.1).

DNA concentration (µM)	Max. DNA:vesicle ratio 100 nm LUVs	Max. DNA:vesicle ratio 200 nm LUVs	Max. DNA:vesicle ratio GUVs (r = 5.8 µm)	Max. DNA:lipid ratio LUVs	Max. DNA:lipid ratio GUVs
0.25	$3.6{\cdot}10^{1}$	$1.4{\cdot}10^{2}$	$7.1{\cdot}10^{6}$	$7.8{\cdot}10^{-4}$	$1.2{\cdot}10^{-2}$
0.5	$7.2{\cdot}10^{1}$	$2.9{\cdot}10^{2}$	$1.4{\cdot}10^{7}$	$1.6{\cdot}10^{-3}$	$2.3{\cdot}10^{-2}$
1	$1.4{\cdot}10^{2}$	$5.7{\cdot}10^{2}$	$2.9{\cdot}10^{7}$	$3.2{\cdot}10^{-3}$	$4.8{\cdot}10^{-2}$
2.5	$3.6{\cdot}10^{2}$	$1.4{\cdot}10^{3}$	$7.1{\cdot}10^{7}$	$7.8{\cdot}10^{-3}$	$1.2{\cdot}10^{-1}$
5	$7.2{\cdot}10^{2}$	$2.9{\cdot}10^{3}$	$1.4{\cdot}10^{8}$	$1.6{\cdot}10^{-2}$	$2.3{\cdot}10^{-1}$

Table 5.1 Maximum DNA:vesicle and DNA:lipid ratios for the vesicles used in this project. GUV ratios were calculated by using a GUV radius of 5.8 µm, the average GUV radius of the experiment that tested the effect of DNA concentration on DNA incorporation (Subsection 4.3.4). For DNA:lipid ratios of the vesicles, it was assumed that DNA can only bind to the outer leaflet. Estimates were made using the liposome calculator developed by the Adamala Lab [104].

The majority of studies discussed in chapter 2 studied LUV-LUV fusion with vesicles diameters of ~100 nm. In these studies, they generally used ten to thousand DNA strands per vesicle. These values correspond to the maximum DNA:vesicle ratios of LUVs used in this project (Table 5.1). Since we used a reversible membrane anchor, however, the actual number of DNA strands per vesicle was lower. Through combined quartz crystal microbalance with dissipation monitoring (QCM-D) and spectroscopic ellipsometry (SE) measurements, Van der Meulen et al. found a DNA surface density of ~1.25 pmol/cm^2 on an SLB in equilibrium. They used stearyl as a membrane anchor, but since they observed similar binding kinetics for stearyl and cholesterol, they assumed that the equilibrium surface density of the two membrane anchors is similar. When translating the reported surface density for SLBS to the amount of membrane area we had in a GUV sample, we find a coverage of $3.2{\cdot}10^{6}$ DNAs per GUV.[1] Van der Meulen et al. used a DNA concentration of 2 µM DNA. If the surface density of our GUVs was comparable to the value found for SLBs, the maximum number of DNAs per GUV in Table 5.1 are thus an overestimation by one order of magnitude.

We found that the level of DNA incorporation increased for higher DNA concentrations up to a concentration of 2.5 µM. To describe this behaviour, we fitted a Langmuir adsorption model to the average level of DNA incorporation at specific DNA concentrations, where we found a dissociation constant K_D of 1.12 µM. In earlier work, dissociation constants of 17 nM [95] and 80 nM [96] were found for the adsorption of cholesterol-tagged DNA to an SLB. Our results thus show a dissociation constant two orders of magnitude higher than the cited articles. Literature showed differences in lipid diffusion rates between SLBs and free-standing planar bi-layers [105] or GUVs [106], which is attributed to the lubricating characteristics of the aqueous membrane space of SLBs. Possibly, the difference in measured dissociation constant can be explained in a similar way. It should also be taken into account that the previously reported values were obtained with quartz QCM-D, whereas our results are based on semi-quantitative epifluorescence measurements, thereby complicating quantitative interpretation. We hypothesise that the higher dissociation constant we measured is at least partly caused by vesicle porosity allowing for double leaflet DNA incorporation. We especially observed double leaflet DNA incorporation for higher DNA concentrations. In samples with 5 µM DNA, the highest DNA concentration we tested, one DNA molecule could theoretically insert into a vesicle for every five lipid molecules

[1] We estimated the total area of our GUVs in a single sample to be $2.23{\cdot}10^{2}$ mm^2 per concentration tested using the liposome calculator developed by the Adamala Lab [104].

(Table 5.1). Although the actual DNA density is lower due to saturation and reversibility, we still expect a high membrane coverage of DNA for a concentration of 5 µM, which could lead to strong DNA-DNA interactions disrupting the GUV membrane. Membrane porosity could also be the result of pore formation induced by desorption of the reversible membrane anchor we used [62].

5.3 GUV-LUV fusion efficiency peaks at 1 µM DNA

We found that GUV-LUV binding and fusion increased for up to 1 µM DNA. This is in line with the majority of studies discussed in Subsection 2.9.3, who found a lower limit dependence on DNA density for lipid and content mixing [61, 65, 67]. Next to this, we found that GUV-LUV binding and fusion decreased for DNA concentrations higher than 1 µM. We hypothesise that this was caused by ssDNA blockage and self-dimerisation. An upper limit of DNA density was also found for SLB-LUV fusion by Simonssen et al. [67], who mention steric hindrance and electrostatic repulsion as possible explanations for the observed fusion inhibition at higher concentrations. An argument for steric hindrance was also provided by Lengerich et al. [69], who reported that in the case of tBLM-SUV fusion, vesicle mobility on the tethered bi-layer decreased for higher DNA densities. In our case, this could mean that GUV-LUV binding and fusion is impeded for higher DNA concentrations due to decreased LUV mobility along the GUV membrane. However, steric hindrance would require a high membrane surface density of DNA. This for example follows from studies on ezrin-mediated actin binding on SLBs, where actin filaments shorter than 1.5 µm were still able to freely diffuse at an actin concentration of 5 µM [107]. Assuming the $3.2{\cdot}10^6$ DNAs per GUV described in Section 5.2, we find a DNA:lipid ratio of $5.3{\cdot}10^{-3}$, which corresponds to one DNA molecule for every ~190 lipids. This in turn means that the distance between individual DNA molecules on the GUV membrane is ~6.5 nm.[2] The anti-parallel binding of the DNA used required the 200 nm LUVs to be brought close to the GUV membrane to allow for binding. It is therefore likely that the DNA density on our GUVs was sufficiently high for steric hindrance to be a limiting factor for binding.

We used a lower lipid concentration to form GUVs compared to LUVs. Consequently, the maximum DNA:lipid ratios for GUVs are higher than they are for LUVs (Table 5.1). We expect that packing of DNA on the GUV membrane caused steric hindrance, thereby preventing GUV-LUV binding. It would therefore be interesting to study the effects of varying DNA coverage on GUV membranes while keeping DNA density on LUVs constant in future experiments. We also found that the presence of 100 mM KCl enhances GUV-LUV binding compared to a KCl concentration of 25 mM. This is in agreement with earlier work, where Morzy et al. showed that ~100 mM monovalent cations are required to maximise DNA incorporation at a pH of 7.5, which is within the acidic range of living systems [73]. This concentration of monovalent cations should thus be used to maximise fusion efficiency.

In conclusion, we found the highest levels of GUV-LUV binding and fusion for a DNA concentration of 1 µM. However, we found no strong correlation between the LUV signal on the GUV membrane and the HPTS ratio. This could be caused by a large fraction of bound vesicles not transitioning towards complete fusion [35], and/or by limitations of epifluorescence microscopy (Section 5.10).

5.4 Coupling DNA to tension to enhance fusion efficiency

Earlier studies showed the effective fusion of vesicles with a tension-mediated approach [e.g. 30, 48]. To enhance fusion efficiency, we coupled our DNA-mediated fusion approach with this fusion strategy based on membrane tension. We studied the effect of membrane tension on DNA incorporation, and DNA-mediated GUV-LUV binding and fusion to investigate if the two fusion methods are compatible with each other. Here, we varied membrane tension by changing osmotic conditions from superhypotonic (Δ_{Osm} ≈ -25 mOsm) to superhypertonic (Δ_{Osm} ≈ 45 mOsm) conditions. We found no significant effect of osmotic conditions on DNA incorporation. Putting GUVs under tension via a transient osmotic shock (Δ_{Osm} ≈ -30 mOsm), however, drastically increased the fusion rate from 18% to 30%. Interestingly, more GUV-LUV binding was observed for slightly hypertonic conditions (Δ_{Osm} ≈ 10-15 mOsm), where we expect vesicles to deflate. To further enhance the efficiency of our fusion protocol, it could

[2] This was calculated by considering a GUV membrane as a collection of adjacent circles, where a DNA molecule is present in the centre of each circle. Here, we adopted a surface area of 0.7 nm^2 per PC head group [104].

thus be beneficial to first adopt hypertonic conditions when GUVs and LUVs are mixed, followed by the transition towards hypotonic conditions. It should be noted, however, that the increase in fusion rate for hypotonic conditions was way more dramatic than the increase in GUV-LUV binding for hypertonic conditions. We therefore conclude that hypotonic conditions are more advantageous to enhance fusion efficiency.

Our findings are reminiscent of earlier work from Miller et al. [108], who found increased fusion of liposomes (d ~ 40 nm) under hypertonic conditions, but who had to convert to hypotonic conditions to promote subsequent fusion of the fusion products. The authors hypothesised that high membrane curvature of the small liposomes was the driving force for the first fusion events. Once the size of fusion products increased, membrane curvature decreased. The realisation of subsequent fusion events therefore required increased membrane tension. In our case, we hypothesise that the increased detection of GUV-LUV binding under hypertonic condition was caused by osmotic deflation. Here, GUV membrane fluctuations could increase the maximum contact area between LUVs docked to GUVs, thereby increasing the chance to form a hemifusion stalk. However, we are unsure if this would indeed lead to an increased LUV signal on the GUV membrane.

One issue with tension-mediated fusion is that membrane tension can greatly vary among GUVs, possibly caused by unequal encapsulation of solvents. According to Lira et al. [52], membrane tension can range from 10^{-9}-10^{-3} N/m among vesicles within a single sample, where membrane tension was determined through electrodeformation [109, 110] or membrane fluctuation analysis [111]. This could also explain why the majority of our GUVs remained unfused. To study the variety in membrane tension among GUVs, one could make use of the tension-dependent lipid dye Flipper-TR® in future experiments [112]. This lipid dye senses mechanical changes of lipid bi-layers, and could therefore be used to relate membrane tension to vesicle fusion on a population level by fluorescence-lifetime imaging microscopy (FLIM) imaging.

Osmotic differences used to study the effect of membrane tension on vesicle fusion vary greatly among studies. Deshpande et al. [30], for example, only used osmotic differences of a few mOsm, since they state that membranes disrupt for larger differences. Arribas Perez and Beales used osmotic differences of ±10 mOsm [48], and we even introduced an osmotic shock of ~-30 mOsm in our fusion protocol. Although we observed some background fusion, which we think was partly caused by vesicle leakage, only ~5% of vesicles contributed to this background fusion. Next to this, we observed a background fusion rate of ~1% for the sample without osmotic shock, suggesting that potential vesicle leakage was not fully induced by the osmotic shock. Our results also indicate that rupture-reseal events, which were attributed to be the limiting factor for fusion by Deshpande et al. [30], were not limiting fusion to a great extent in our case. If this would have been the case, we would have observed more vesicles with an internal HPTS signal.

5.5 The versatility of our fusion protocol

To be compatible with other synthetic cell modules, the fusion protocol should be able to operate in a wide range of conditions, including vesicles produced with different GUV formation methods and varying membrane compositions. We therefore showed that our fusion protocol is compatible with PE-containing and physically relevant GUVs. The latter is of specific interest in the context of an integrated synthetic cell. Corresponding to earlier work [39], we showed that GUV-LUV binding was promoted for PE-containing GUVs, but that it resulted in a lower fusion efficiency compared to DOPC GUVs. The PE-containing lipid composition we tested consisted of DOPC/POPE in a 7:3 molar ratio. A lipid composition that is frequently used in fusion studies is DOPC/DOPE/cholesterol in a 2:1:1 molar ratio [e.g. in 59, 61–63, 65, 66]. Possibly, the fusion efficiency of our fusion protocol can be further enhanced by adopting this lipid composition. Here, we expect an increased fusion rate facilitated by a larger fraction of lipids that exhibit negative spontaneous curvature and phase segregation behaviour [59, 71].

The proposal of a hydrophobic defect as intermediate state between vesicle docking and hemifusion (see Section 2.6), where both fusing membranes are pointed towards each other, suggests that both membranes require negative curvature to form a hemifusion stalk [41]. Next to varying the lipid compo-

sition of GUVs, it would thus be interesting to vary the lipid composition of LUVs in future experiments. This would also allow for adaptations of the membrane donor independent of the GUV, which would be interesting in the context of a synthetic cell. Here, it is convenient that most studies on the effect of lipid composition on vesicle fusion have been performed with LUVs.

Next to the advantage of negative curvature for vesicle fusion, literature has pointed out the need for positive curvature for fusion pore formation [35]. In a future experiment, it would be interesting to form vesicles containing positive and negative curvature generating lipids, i.e. using both cone-shaped and inverted cone-shaped lipids. Possibly, positive curvature could be delivered to the hemifusion stalk through lipid diffusion in this case, thereby promoting pore formation and thus overcoming the hemifusion stalk.

Apart from membrane curvature, Chan et al. [66] showed that fusion can be affected by the presence of lipid dyes, possibly caused by the physico-chemical properties of the dyes. We showed that our fusion protocol is not substantially influenced by the presence of 0.5 mol% Atto488 in GUV membranes, thus making this a useful dye for our fusion assay. We did observe a lower fusion rate for GUVs containing 0.5 mol% Atto655, the lipid dye we generally used for LUVs in other experiments. Fusion efficiency could thus potentially be increased by eliminating the usage of Atto655.

We tested if our fusion protocol was compatible with eDICE next to gel-assisted swelling, the GUV formation method that was used for other experiments. Here, we found that vesicles formed by eDICE also showed fusion. Although we observed a lower fusion rate for vesicles formed by eDICE (13% vs. 32% for gel-assisted swelling), this was possibly caused by poor vesicle formation. The advantage of eDICE is that it has a higher encapsulation efficiency than the gel-assisted swelling method [31]. It is therefore a suitable method to employ GUV-LUV fusion as a delivery mechanism. Here, one could for example encapsulate actin monomers in GUVs and an actin nucleator in LUVs. The formation of an actin cortex upon mixing GUVs and LUVs would be an elegant future proof-of-principle experiment showcasing the versatility of our fusion protocol.

5.6 Indications of membrane growth for fusion with 200 nm LUVs

Vesicle extrusion through small pores can be problematic, due to the high forces required for this process. Earlier studies generally performed 21 extrusion steps to obtain SUVs [e.g. in 30, 62]. However, we showed that four extrusion steps are sufficient to acquire SUVs close to their minimum size. In this way, we demonstrated that existing methods for SUV fusion can be simplified without sacrificing fusion efficiency. Since we detected low fusion rates using 30 nm SUVs, probably caused by the fact that they deliver a low amount of HPTS to a GUV per fusion event, we only used 100 and 200 nm LUVs for DNA and tension-based experiments. For these LUVs, on the other hand, we found comparable fusion rates of 25% and 23%, respectively. Here, sizes of LUVs were verified with DLS measurements. Since 100 nm LUVs had to fuse more than 200 nm LUVs for fusion to be detectable, our results confirm earlier findings stating that smaller vesicles are more fusogenic [42, 43].

For the samples showing the highest fusion rates, we generally found a significant difference in average GUV radius between fused and unfused vesicles. This difference could partly be explained by the swelling of tense vesicles, but this can only increase the surface area of a GUV with ~5% [102]. Next to this, fused GUVs were often elongated or fluctuating. Based on these observations, we hypothesise that the differences observed in mean GUV radius are the result of membrane growth. However, this observation should be verified in future experiments by observing live fusion.

For four independent experiments using 200 nm LUVs (all with 1 µM DNA and an osmotic shock), we found an average increase in DOPC GUV radius of 35%, which would correspond to a striking ~1.8-fold increase in membrane surface area. This is is close to the duplication in membrane area required for sustained synthetic cell division. If the increase in average radius is indeed the result of membrane growth, this corresponds to an average number of $3.5 \cdot 10^3$ GUV-LUV fusion events for fused vesicles, where we took the average GUV radius of the four independent experiments with DOPC GUVs (r = 6.6 µm). For GUVs with the same conditions but a physiologically relevant lipid composition, we found a

comparable difference in average GUV radius of 37% for fused vesicles, indicating the applicability of the fusion protocol in the context of a synthetic cell. Although we found that 100 nm LUVs are more fusogenic than 200 nm LUVs, we found no difference in average radius between fused and unfused GUVs mixed with 100 nm LUVs. This suggests that 100 nm LUVs are too small to sufficiently increase membrane surface area with our fusion protocol.

5.7 Finding the optimal balance between fusion rate and membrane growth

Fused GUVs showed a significant increase in mean GUV radius both in absence and presence of an osmotic shock. Although vesicles with osmotic shock showed an increased fusion rate, the average GUV radius increased more for fused GUVs without osmotic shock (52% vs. 41%). We hypothesise that this was caused by vesicle rupture of the most tense GUVs in the osmotic shock sample (i.e. the vesicles we expect to be most fusogenic), thereby losing the vesicles that had the potential to exhibit most membrane growth. The largest increase in average GUV radius (66%) was found for fused vesicles with osmotic shock and a DOPC/POPE lipid composition in a 7:3 molar ratio. Although we found a lower fusion rate for these vesicles compared to vesicles consisting only of DOPC (23% vs. 32%), the results suggest that individual GUVs containing cone-shaped lipids have the ability to undergo enhanced levels of fusion. Possibly, the lower fusion rate for PE-containing GUVs is caused by an unequal incorporation of the cone-shaped lipids among vesicles.

We thus found lower fusion rates for the samples showing the highest level of membrane growth. This indicates that, while fewer GUVs undergo fusion, they grow more if they do fuse. Since the membrane surface area for the samples exhibiting most membrane growth increased even more than required for sustained synthetic cell division (~2.3 and ~2.8-fold), it would be interesting to find an optimal balance. At this optimal balance, membrane surface area doubles and fusion rate is maximised. Future studies should thus focus on the relationship between fusion rate and membrane growth, and how this is affected by factors such as osmotic conditions and membrane composition.

5.8 Future optimisation of the fusion protocol

5.8.1 Timescales of fusion steps

We studied the timescales of two steps of the fusion protocol: DNA incorporation and GUV-LUV binding. In agreement with earlier work on cholesterol-tagged DNA adsorption on SLBs [96], we found that DNA readily incorporates in GUV membranes within a time frame of minutes. Next to this, we found that the level of GUV-LUV binding is close to its maximum value after the first time point we measured at t = 10 min. We did not asses the timescale of fusion, and generally incubated vesicles for 60-80 minutes to allow for fusion. Other studies showed that vesicle docking, lipid mixing and vesicle fusion occurs on the timescale of seconds for DNA-mediated SLB-LUV and tBLM-SUV fusion [67, 69]. It is therefore likely that the current time steps of the fusion protocol can be shortened to obtain fusion rates similar to our reported values.

5.8.2 ssDNA sequence

At the end of our project, we found out that one of the strands adopted from Dreher et al. was erroneously displayed in their paper [16], causing the strands to be not fully complementary. Using the OligoAnalyzer developed by Integrated DNA Technologies [97], we determined the heterodimers our ssDNAs are able to form, resulting in a proximal binding (Figure 5.1a) or distal binding state (Figure 5.1b). Since the differences in free energy (Delta G) of these binding states are comparable, we expect that both were present in our experiments. We would have had optimal binding if both strands were fully complementary (Figure 5.1c). The difference in free energy of the optimal binding state is approximately twice as large as the other two binding states, leading to a stronger bond. Next to this, the optimal binding state brings the LUV slightly closer to the GUV membrane, which was shown to enhance fusion efficiency [66]. This is because thermally induced fluctuations have a better chance to result in fusion when the involved membranes are closer to each other. Although we still obtained significant fusion rates, it would be interesting to assess if fully complementary strands result

in even more fusion in future experiments. Another interesting approach would be to utilise poly A/T sequences, which have been shown to increase lipid and content mixing for LUV-LUV fusion [65]. This is probably caused by their ability to bind while only partially overlapping, thereby giving them more geometrical flexibility.

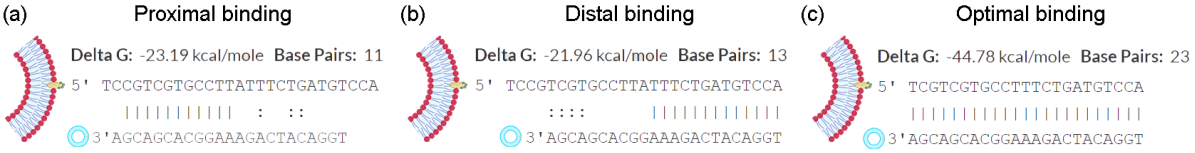


Figure 5.1 Dimerisation states of the partially complementary and fully complementary ssDNAs. Due to the partially complementary ssDNAs used in the project, we could only achieve proximal binding (a) or distal binding (b). Optimal binding would have required fully complementary strands (c). chol-DNA2-x was inserted in the GUV membrane (red), whereas x-DNA1-chol was inserted in the LUV membrane (cyan). Delta G indicates the difference in free energy (kcal/mole). Solid lines indicate base pairs that contributed to Delta G. Base pairs indicated by dashed lines could be formed as well, but did not contribute to Delta G. Dimerisation states were determined with the OligoAnalyzer developed by Integrated DNA Technologies [97]. Figure made with BioRender.

5.8.3 Membrane anchor

As mentioned in Section 5.2, earlier studies found that cholesterol-tagged DNA binds reversibly to SLBs [95, 96]. Although we described indirect observations suggesting that DNA bound reversibly to our GUVs as well, we did not directly observe this. One could measure the desorption rate of cholesterol-tagged DNA by giving it a fluorescent tag, and by subsequently following its signal on the GUV membrane upon dilution. Next to this, fluorescence recovery after photo-bleaching (FRAP) could be used to determine DNA-turnover on the membrane. Although we did not directly study DNA incorporation in LUVs, it makes sense to assume that cholesterol-tagged DNA binds reversibly to these vesicles as well. This assumption could be verified by performing a FRET assay, where cholesterol-tagged DNA is endowed with a donor dye and the LUV membrane with an acceptor lipid dye [62]. Similar to the GUV experiment, one would expect a decrease in FRET signal upon dilution of the LUV sample.

We hypothesise that the reversible binding of cholesterol-tagged DNA explains why fusion is enhanced for the working fusion protocol. In the ineffective fusion protocol, GUVs and LUVs were consecutively added to quenching OBS (typically in a 1:1:2 volume ratio), thereby introducing a 4x dilution of the cholesterol-tagged DNA. Given the reversibility of binding, this dilution led to the introduction of a new equilibrium state with a lower equilibrium surface coverage. In the working fusion protocol, however, GUVs and LUVs were incubated with each other 1:1 before they were added to quenching OBS, thus involving only a 2x dilution of both DNA types. We postulate that in this case, the DNA membrane density was optimal for efficient fusion. This is in line with the increased level of GUV-LUV binding we observed for the working fusion protocol compared to the ineffective fusion protocol.

Although vesicles were observed in quenching OBS for the working fusion protocol as well, meaning that cholesterol-tagged DNA was diluted 4x in the final sample composition, we can assume that incorporated DNA was stabilised in the membrane through hybridisation with the complementary strand. Once docked, GUV-LUV bonds could also become stronger over time by the recruitment of additional DNA strands, thus no longer being affected by the desorption of a single DNA strand. Finally, the increased detection of GUV-LUV binding for the working fusion protocol could be explained by its enhanced fusion efficiency: once LUV lipid dye was incorporated into the GUV membrane upon fusion, its fluorescent signal became independent of anchor reversibility.

If cholesterol anchors are indeed stabilised upon GUV-LUV binding, the rapid transfer of vesicles after a short GUV-LUV incubation step would theoretically allow us to observe life fusion. However, since it is reported that fusion occurs on the timescale of seconds [67, 69], we expect that preparing the sample for imaging will take longer than fusion. As shown by previous work, the reversibility of DNA incorporation could be overcome by using a bivalent cholesterol anchor [95] or a di-stearyl anchor

[96]. Eliminating anchor reversibility would not only allow for the observation of live fusion, but it could also enhance fusion efficiency: more incorporated DNA would remain available for vesicle binding, and less unincorporated DNA would be able to block vesicle binding. In this way, the decreasing trend we observed for GUV-LUV binding for higher DNA concentrations could possibly be mitigated or even eliminated, depending on the contribution of ssDNA blockage to this effect. Corresponding to this, we described that irreversible anchors have been found to enhance lipid and content mixing for 100 nm LUVs in Chapter 2 [61, 62]. Utilising an irreversible anchor could thus be beneficial to acquire more membrane growth. In the context of a synthetic cell, however it might be more beneficial to use a reversible membrane anchor. As shown by earlier work, cholesterol-tagged DNA can be readily removed from membranes by performing a washing step with buffer [95, 96]. This would allow scientists to reset a vesicle membrane after membrane growth, after which it could be exposed to another growth cycle.

Finally, we hypothesise that using a reversible membrane anchor is less problematic for GUV-LUV fusion than it is for LUV-LUV fusion. In Chapter 2, we described that LUV-LUV lipid and content mixing is often enhanced by using irreversible or longer anchors. In LUV-LUV fusion studies, a single LUV has to (hemi)fuse with another LUV to be able to detect this event. In our case, it is likely that many GUV-LUV fusion events were required to detect fusion, due to the dramatic difference in size between the two vesicle types (Section 5.1). Next to this, the larger size of GUVs causes them to be less fusogenic than LUVs. Still, we found a relatively high fusion rate of ~30% using a single cholesterol anchor. Possibly, cholesterol-tagged DNA is more stably incorporated in GUV membranes than in LUV membranes since they have a lower membrane curvature.

5.9 Charge-based experiments

Next to fusion experiments based on DNA and tension, we performed charge-mediated GUV-SUV binding and fusion experiments (Appendix A). We found that the fractions of charged lipids used (20 or 40 mol%) did not influence the measured size of SUVs with DLS experiments. Furthermore, we observed significant binding of 20 mol% TAP SUVs to 40 mol% PS GUVs, whereas no significant binding was found for 40 mol% TAP SUVs to 40 mol% PS GUVs. We hypothesise that 40 mol% TAP SUVs more rapidly neutralised GUVs upon GUV-SUV binding than 20 mol% TAP SUVs, thereby preventing the occurrence of additional binding events [52]. Here, we assume that the level of GUV-SUV binding involving 40 mol% TAP SUVs was too low for us to detect it. Using 20 mol% TAP SUVs and 40 mol% PS GUVs, we sporadically observed fused GUVs. In general, charge-based experiments were often accompanied with vesicles sticking to each other and to the surface of the microscopy chamber. For these reasons, we concluded that DNA and tension-mediated fusion were more beneficial for us as a strategy to achieve membrane growth.

5.10 Research limitations

One of the limitations of this study is that we used epifluorescence microscopy. Although this technique provided us with valuable insights in DNA and tension-mediated fusion, we could only employ it for semi-quantitative analysis, partly caused by the blur from out of focus objects that is typical for widefield microscopy [113]. We therefore recommend to perform future experiments with confocal microscopy to improve the signal-to-noise ratio. By using this technique where light traverses through a pinhole, thereby minimising out of focus signal, Mora et al. quantified lipid mixing for GUV-LUV fusion [82].

It should also be noted that the majority of our experiments were performed a single time. Although they generally involved quite large numbers of GUVs per observed sample (typically in the order of 10^2), repeats of our experiments are required to safeguard quantitative reproducibility.

Another limitation of this study is that porosity of LUVs was not taken into account. Transient pore opening of LUVs that were hemifused with GUVs might have resulted in false positives for fusion. Chan et al. for example reported that vesicles can exchange contents through flickering fusion pores [66]. Lengerich also reported this issue for tBLM-SUV fusion, but they presumed that its contribution to false positives for fusion is small [69]. Leakage of unbound LUVs, on the other hand, could have

led to an underestimation of the fusion rate. In this case, DPX could have leaked into LUVs, thereby delivering no fluorescent molecule upon GUV-LUV fusion. Malinin et al. found ~5% of 120 nm LUVs to be leaky for PEG-mediated fusion at an osmotic difference of 50 mOsm [42], which is an osmotic difference comparable to what our LUVs experienced during the osmotic shock. However, the leakiness could also be caused by PEG-membrane interactions. To determine if and how LUV leakiness influenced our results, we recommend to investigate the degree of LUV porosity in future experiments. This could for example be done in bulk by encapsulating a fluorescent dye in LUVs and by adding its quencher to the outer solution, to subsequently measure the changes in fluorescent signal [61].

Since we used a reversible membrane anchor, it is in theory possible that DNA shuttled from GUV membranes to LUV membranes or vice versa. We can therefore not fully rule out the possibility that GUVs fused with GUVs or that LUVs fused with LUVs in our experiments. However, we expect that upon mixing GUVs with LUVs, incorporated DNA was rapidly stabilised through DNA hybridisation (Subsection 5.8.3), thereby making GUV-GUV or LUV-LUV fusion exceedingly unlikely. Furthermore, if GUV-GUV fusion did frequently occur, we would expect to have observed many hemifused GUVs as well, which we did not. The delivery of HPTS by LUVs to a GUV was required to detect fusion. In the unlikely case that GUV-GUV fusion did occur, we therefore do not expect that these events were classified as fused vesicles by our method, and thus assume that they did not skew our reported fusion rates.

A final limitation of this study is that we did not observe live fusion, which would be insightful to study membrane growth for individual GUVs, for example by using an MPA set-up. We performed the first steps to realise this experiment by showing no changes in GUV area under constant pressure for aspirated GUVs, by visualising LUV localisation on the membranes of GUVs in bulk after locally injecting LUVs, and by the initial development of a novel fusion detection method. By further developing these assays, it could be confirmed that the GUV size increase we observed is indeed the result of membrane growth. Next to this, the MPA set-up would create opportunities to study the influence of membrane tension on vesicle fusion for single GUVs, and to study vesicle shape transitions upon GUV-LUV fusion.

6
Conclusion

In this study, we presented a novel GUV-LUV fusion protocol based on a coupled DNA and tension-mediated approach. We used this protocol to generate membrane growth for GUVs, which is required for a synthetic cell to divide. We adopted a content mixing assay to detect fusion, where we encapsulated the fluorescent dye HPTS in LUVs, whereas we quenched the outer solution with DPX. A fluorescent signal from HPTS was subsequently only visible from the interior of GUVs upon successful vesicle fusion. We determined fusion rates by calculating the fraction of GUVs exhibiting content mixing, where we found a substantial fusion rate of ~30% under optimal conditions. For no DNA controls, we detected ~5% background fusion that was caused by DNA-independent fusion or vesicle leakage.

We found that DNA membrane incorporation increased with DNA concentration, with DNA density on the membrane saturating above 2.5 µM DNA in solution. For GUV-LUV binding and fusion, on the other hand, we found that the rate of these processes strongly increased up to 1 µM DNA, but decreased for higher DNA concentrations. The decrease in fusion rate was more prominent than the decrease in GUV-LUV binding at these higher DNA concentrations. We hypothesise that GUV-LUV binding and fusion peaked at 1 µM DNA due to ssDNA blockage, steric hindrance, and DNA self-dimerisation.

In agreement with earlier work, we found decreased levels of GUV-LUV binding for a lower KCl concentration. Our findings suggest that fusion is enhanced for higher levels of GUV-LUV binding as well, but we found no strong correlation between LUV signal on the membrane and fusion rate. This was possibly caused by many LUVs being trapped in a vesicle docking or hemifusion state. Another option is that the epifluorescence microscopy technique we used was limited in quantifying the level of GUV-LUV binding. To gain more insights in the mechanics of GUV-LUV fusion, we recommend to perform future experiments with confocal microscopy.

To assess if fusion strategies based on membrane tension and DNA hybridisation are compatible with each other, we investigated the effect of membrane tension on the different steps of DNA-mediated fusion. We did this by varying osmotic conditions during DNA incorporation, DNA-mediated GUV-LUV binding, and DNA-mediated fusion. Here, we found no effect of membrane tension on DNA incorporation. GUV-LUV binding was slightly improved for hypertonic conditions, whereas fusion efficiency dramatically increased for hypotonic conditions (from 18% to 30%). These results showcase that DNA-mediated fusion can be enhanced greatly by combining it with a tension-mediated approach.

We demonstrated the versatility of our fusion protocol by assembling GUVs with different membrane compositions and formation methods. Firstly, we showed that not only simple DOPC GUVs, but also GUVs with more complex and physiologically relevant membrane compositions could undergo fusion. Secondly, we showed that the technique can be applied successfully to both GUVs formed by gel-assisted swelling and eDICE. In this way, the presented approach is compatible with an integrated synthetic cell, and allows for experiments where LUVs can be employed to deliverer specific compounds to GUVs.

Earlier work showed that using specific lipid dyes can influence lipid mixing rates, but we found no substantial effect of the GUV lipid dye used for fusion experiments on fusion efficiency. We found increased levels of GUV-LUV binding when we included the cone-shaped lipid POPE in GUVs, but no enhanced fusion efficiency. Incorporation of cholesterol next to PE-containing lipids could possibly further increase fusion rate, by a larger fraction of lipids exhibiting negative spontaneous curvature and

phase segregation behaviour. Next to this, it would be interesting to vary lipid composition of LUVs in future experiments. Another way to increase the fusion rate could be to decrease the degree of DNA coverage on GUV membranes while keeping the DNA density on LUV membranes the same, possibly decreasing the levels of steric hindrance and self-dimerisation. Furthermore, the employment of an irreversible membrane anchor could boost fusion efficiency.

Corresponding to earlier results stating that smaller vesicles are more fusogenic, we found that 100 nm LUVs were more prone to fuse with GUVs than 200 nm LUVs. Here, sizes of LUVs were verified with DLS measurements. For the samples with the highest fusion rates, we generally found a significantly larger population-averaged GUV radius for fused vesicles compared to unfused vesicles, suggesting that these vesicles exhibited membrane growth. Mean GUV radii of fused vesicles were 23-66% larger than mean radii of unfused vesicles for these samples, which would correspond to a striking ~1.5-2.8-fold increase in membrane surface area. However, to confirm that it is indeed membrane growth we observed, future experiments observing live fusion should be performed. We performed initial experiments for the development of a new fusion protocol, involving the strong binding affinity between His-tagged GFP And nickelated lipids, that would potentially allow for the observation of live fusion.

No difference in mean radius was found between fused and unfused GUVs exposed to 100 nm LUVs, suggesting that these LUVs were too small to facilitate membrane growth. We demonstrated that both DNA incorporation and GUV-LUV binding were close to their maximum levels within the first time points measured at t = 5-10 minutes. Since we used a subsequent vesicle incubation step of 60-80 minutes to allow for fusion, the protocol allows for GUV-LUV fusion within a time frame of two hours. The duration of the protocol can possibly be shortened further by reducing the time of vesicle incubation.

We also performed charge-based experiments, but due to a low fusion efficiency and aberrant vesicle structures, we did not further develop this method as an approach to acquire membrane growth. In other experiments, we showed that GUVs aspirated with an MPA set-up show minimal changes in surface area under constant pressure. Furthermore, we were able to locally inject LUVs to GUVs in bulk, leading to the rapid localisation of LUVs on the GUV membrane. These findings can be combined in novel experiments to study the effect of membrane tension on fusion of single GUVs, as well as the shape transitions that GUVs undergo during the process of membrane growth. Here, a single GUV would be trapped in an MPA set-up, where membrane tension could be controlled by the application of negative pressure. Simultaneously, LUVs could be injected to the local environment using an additional micropipette.

This work is the first systematic study of the discussed parameters in GUV-LUV fusion. We demonstrated the efficacy and broad applicability of our optimised fusion protocol, opening the door to its application for *in vitro* membrane growth. Essentially, these new insights will allow scientists to combine our efforts towards membrane growth with the successful division of a synthetic cell in the future.

Part II

Building the Foundation of a Synthetic Cell Innovation Ecosystem

7

Introduction

Synthetic cells are believed to drastically change both our understanding and utilisation of biology in the 21st century. Although the research into synthetic cells is currently still highly fundamental, it is believed that they can lead to revolutionary applications in the future [9]. The initial steps to explore these revolutionary applications are undertaken by the Synthetic Cell Initiative (SynCellEU), a European collaboration that currently consists mainly of researchers, but has the long-term ambition to form strong collaborations with industry and society. Similar to BaSyC, SynCellEU was established in 2017. It was formed by Delft University of Technology (TU Delft), the University of Oxford, the Max Planck Institute of Biochemistry, and the French Alternative Energies and Atomic Energy Commission (CEA) [114]. The CEA is an organisation that is active in many different fields, from defence and security to fundamental research in the natural sciences [115]. The CEA works together with many academic and industrial partners, thereby creating a bridge between these two stakeholder groups. SynCellEU is currently managed by small budgets from all founding parties. Next to this, the initiative is supported by the Kavli Foundation. The Kavli Foundation is an organisation that funds research to improve society, supporting scientists from four different disciplines: astrophysics, nanoscience, neuroscience and theoretical physics [116]. An overview of the logos of the founders and sponsors of SynCellEU is visible in Figure 7.1.

Figure 7.1 An overview of organisations that founded and/or support SynCellEU.

BaSyC and SynCellEU have the same Programme Manager, dr. Stefania Usai. She is affiliated to TU Delft, similar to the Communication Officers of both programmes (Heleen van Rooijen-Bosscha for BaSyC and Celine Alkemade for SynCellEU). The common origin of both programmes is also visible through the resemblance of their logos (Figure 7.2). One of the differences between the two programmes is that BaSyC mainly focuses on performing fundamental research, whereas SynCellEU is also engaged in exploring potential future applications of a synthetic cell. They do this by creating a European platform where people from academia, industry and governmental organisations can join their forces to build a synthetic cell from scratch. On the long term, SynCellEU aims to build a European innovation ecosystem, where universities, industry, and governmental organisations develop synthetic cell applications in a joint effort through co-production. Next to establishing a European innovation ecosystem, SynCellEU intends to address the societal impact that synthetic cells may have in the future. It does so by performing discussions focused on ethical issues and responsible research and

innovation (RRI). In this way, it aims to create a “societal and legal framework for the creation of synthetic cells” [114].

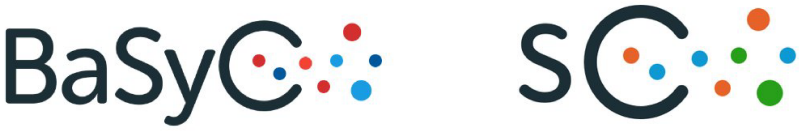


Figure 7.2 The logos of BaSyc (left) and SynCellEU (right). The resemblance of the logos indicates the common origin of the two programmes.

7.1 Theoretical background

7.1.1 The definition of an innovation ecosystem

The concept of an innovation ecosystem has many different definitions. Since the details of the future collaborations that SynCellEU aspires are still unclear (Chapter 9), it is useful to first present an overview of the differences and similarities between definitions of this concept as it is described in literature. In this way, we will become aware of the different options to fill in an innovation ecosystem. Subsequently, we will state the used definition of an innovation ecosystem in this study.

In their systematic literature review, Gomes et al. explored the concept of an innovation ecosystem [117]. For this, they studied 125 articles with a bibliometric and content analysis. This means that they both studied the overlapping themes of articles describing innovation ecosystems, as well as the amount of times specific articles were cited, something that becomes increasingly relevant with the growing number of research articles and the available tools to analyse them [118]. Here, it is assumed that the most-cited articles have the largest impact on the field. Through their analysis, Gomes et al. found a great variety in descriptions of innovation ecosystems, clarifying the difficulty in pinpointing what they exactly are. This does not only result into confusion and disagreements, but can actually cause the meaning of the concept to be scattered, complicating the comparison between different articles. Oh et al. attributed this variety in definitions to researchers fitting a definition to their own benefit [119], and criticised the concept of innovation ecosystems in general due to its “arguably flawed analogy to natural ecosystems” [120].

A trend that Gomes et al. did identify in the analysed articles is a shift in literature, where studies increasingly started to focus on innovation ecosystems rather than business ecosystems. Although some scholars argue that the two ecosystems are the same, Gomes et al. state that literature on business ecosystems mainly focuses on value capture and competition, whereas articles about innovation ecosystems are more about value creation and collaboration. Here, value capture is about creating competitive advantage and maximising profits, while value creation is about developments and activities that are beneficial for consumers and other parties. The authors argue that both value creation and value capture are required for an ecosystem to thrive, but that business and innovation ecosystems each relate to one of the concepts. Next to this, they cite an article by Adner and Kapoor who state that in ecosystems, value capture follows upon value creation [121], which could have important implications for the formation of an innovation ecosystem.

By studying overlapping themes in articles describing innovation ecosystems, Gomes et al. came up with the following definition: *“an innovation ecosystem is set for the co-creation, or the joint creation of value”* [117]. Next to this, they state that innovation ecosystems are centred around a focal firm, which is a for-profit business. Other players such as consumers, suppliers, and managers play a role as well. They recognise that these partners experience both collaboration and competition, and that they co-evolve in their ability to create value.

In another more recent conceptual review, Granstrand and Holgersson start their quest to find a definition for an innovation ecosystem by describing the concepts that it is built up from: systems, innovation, innovation systems and ecosystems [120]. They describe a system as a set consisting of C components, that are associated with each other through R relations. They also highlight the dynamics of an open system, which is mediated by the *"transformation of input into outputs through activities performed by agents or actors interacting with an environment"* [120]. The next concept described is innovation. This concept has a fluid meaning, both in terms of its strict definition and the emotional and imaginative association with it [122]. However, most current definitions see an innovation as the result of a process, where it adds something new that is useful or successful in being applied. These two concepts together form an innovation system, a concept that was first described in the fields of economics and policy sciences.

From selected conceptualisations, Granstrand and Holgersson define an innovation system as follows: *"a set of components and the causal relations influencing the generation and utilization of innovations and the innovative performance"* [120]. An ecosystem is finally described as the conceptualisation of *"the flow of material and energy"* [120]. To further elaborate on this, an article where innovation ecosystems are described with ecology theory is cited. In this article by Shaw and Allen, it is described that an ecosystem can *"[recycle] flows of nutrients along pathways made up of living subsystems which are organised into process-oriented roles"*, and that it *"connects living and non-living subsystems" [123]*.

Granstrand and Holgersson then analysed 21 definitions of innovation ecosystems. Similar to Gomes et al., they found that innovation ecosystems literature puts more focus on collaboration than on competition. Gomes et al. suggested that this conceptualisation arose as a response to the predominant focus on value capture and competition in pre-existing literature on business ecosystems. This response included a more elaborate focus on value creation and collaboration in innovation ecosystem literature. Granstrand and Holgersson, however, oppose this view. To strengthen their claim, they cite an article by James F. Moore: *"In a business ecosystem, companies co-evolve capabilities around a new innovation: they work cooperatively and competitively to support new products, satisfy customer needs, and eventually incorporate the next round of innovations"* [124]. Granstrand and Holgersson mention that this is one of the most cited articles on business ecosystems. According to them, it shows that business ecosystems are presented as a concept where collaboration and competition are equally important, contrary to the view of Gomes et al. They subsequently suggest that the shift from business ecosystems to innovation ecosystems might have put too much emphasis on collaboration compared to competition. They also state that the replacement of artefacts and resources is not or only sporadically mentioned in innovation ecosystems literature, while this actually plays an important role. Not only in natural ecosystems, where species substitute each other through natural selection, but also in artificial ones, where pre-existing technologies are replaced by new ones through creative destruction [125].

Through their analysis on innovation ecosystems literature, and by putting additional focus on competition and the replacement of artefacts, Granstrand and Holgersson came up with the following definition: *"An innovation ecosystem is the evolving set of actors, activities, and artifacts, and the institutions and relations, including complementary and substitute relations, that are important for the innovative performance of an actor or a population of actors"* [120]. Their definition is summarised in Figure 7.3, where it can be appreciated that all innovation ecosystem components interact with themselves and each other. Contrary to Gomes et al., the innovation ecosystems is centred around institutions instead of a focal firm, which makes it more generally applicable. In our case, this means that the innovation ecosystem could be centred around SynCellEU. Next to this, we recognise the importance of collaboration, competition, and substitution of artefacts in the innovation ecosystem. The substitution of artefacts is for example related to the replacement of existing technologies with more sustainable alternatives (Chapter 10), and competition turned out to be an important factor in the development of an innovation ecosystem (Section 11.9). For these reasons, we will adopt the definition proposed by Granstrand and Holgersson.

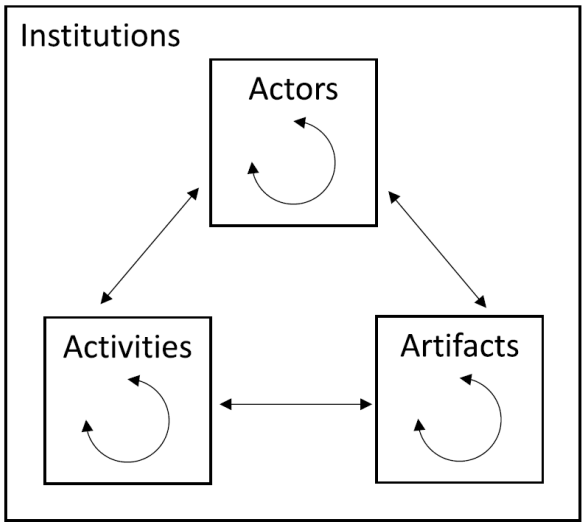


Figure 7.3 Summary of the definition of an innovation ecosystem according to Granstrand and Holgersson [120], from whom the figure was retrieved.

7.1.2 Innovation and collaboration models

In the previous section, we already touched upon the definition of innovation, where we described that it has a fluid meaning. Indeed, a review on innovation processes confirms the ambiguity of the concept, whose definition is often adapted to fit to specific frameworks [126]. Due to its heterogeneous description in literature, it can be insightful to capture the meaning and diversity of innovation processes into descriptive models. Innovation is occurring in the real-world, so one should be able to describe the process by combining the right actors and concepts.

According to the technology-push model, a scientific discovery is turned into an innovation without actually knowing if society desires this innovation [127] (Figure 7.4a). This model stems from the first two decades after the Second World War, when many new applications came to the market due to rapid scientific progress. At the same time, there was a general consensus that science could solve societal issues. As market competition started to increase in the mid 1960s, companies had to find new ways to keep their customers satisfied. Here, companies increasingly started to focus on improving existing technologies, rather than developing new ones. These developments led to the market-pull model, where the innovation process is driven by societal needs (Figure 7.4b). In this model, industry mainly acts as a reactive actor that is driven by the market. Over the years, multiple extensions combining and integrating aspects of both models have been developed [127].

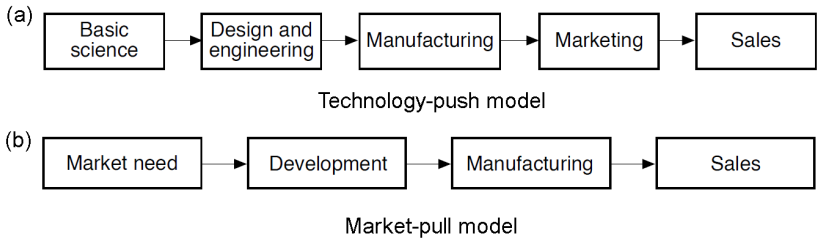


Figure 7.4 The technology-push (a) and market-pull innovation models. Figure adapted from Rothwell [127].

The technology-push model is highly reminiscent, if not the same as the linear innovation model displayed in Figure 7.5a [128]. Here, applications are also developed from basic research without taking the wishes of society into account. This linear description of the innovation process stems from "Mode 1", which describes knowledge production for the sake of knowledge. In Figure 7.5b, a conceptualisation of a non-linear innovation model is displayed. Here, universities, university-related institutions and

firms interact with each other to develop innovations. In this case, university-related institutions perform applied research, and serve as intermediates between universities and companies. This non-linear representation of innovation processes relates to "Mode 2", where multidisciplinary teams collaborate to produce knowledge focused on the application [129]. According to Carayannis and Campbell, linear and non-linear innovation models coexist in innovation ecosystems, which they attribute to be the "Mode 3" form of knowledge production [128].

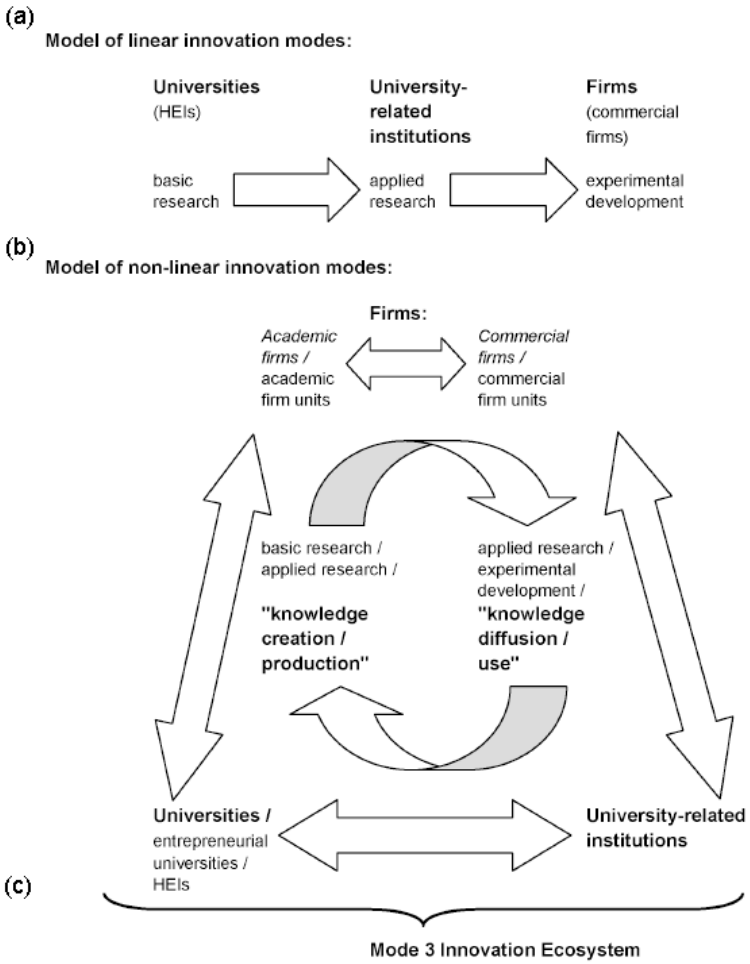


Figure 7.5 Linear (a) and non-linear (b) innovation models are combined into a "Mode 3" innovation ecosystem, where multiple innovation paradigms coexist. Figure adapted from Carayannis and Campbell [128].

Next to relating an innovation ecosystem to innovation models, Carayannis and Campbell relate it to a collaboration model. They specifically relate it to an extended version of the triple helix model. The triple helix model, developed by Etzkowitz and Leydesdorff in the 1990s, describes bilateral collaborations between academia, industry, and governmental organisation [130]. This relates to development of the the non-linear innovation model, where organisation increasingly interact during the development of applications. To describe the interactions between stakeholders in an innovation ecosystem, Carayannis and Campbell proposed the quadruple helix model, where the public is included in the framework [128]. Here, media and culture influence the way in which the triple helix players interact, thereby having an effect on the innovation process as a whole.

7.1.3 RRI elements

Through discussions focused on ethical issues and RRI, SynCellEU aims to address the potential impact of synthetic cells on society [114]. Approaches to assess the societal impact of new technologies

often have their roots in the natural sciences. An example of this can be found in the Human Genome Project (HGP). During this project, where the entire human genome was mapped from 1990 until 2003, the term "ethical, legal and societal implications" (ELSI) was coined [131]. ELSI was an integral part of HGP, and its introduction was related to previous discussions on the risks of technologies such as nuclear energy and pesticides. To prevent that HGP should be suspended due to ethical controversies, the implications of the project were explored in parallel to the execution of it. ELSI has later been adopted in other research projects, for example in the fields of biosciences and biotechnology [132]. However, it has been criticised on its predominant focus on the potential *negative* impact of technological developments [133]. While developing a new technology, it was often assumed that the mere involvement of a social scientist would mitigate potential adverse side-effects. ELSI primarily focuses on the simple linear innovation model (Figure 7.5a), whereas the development of new technologies often involves non-linear relationships (Figure 7.5b). Next to this, ELSI is mainly concerned with research outputs, whereas it pays less attention to research practices. It also assumes a convenient distinction between positive and negative outcomes of research, whereas in reality, the benefits or downsides of research can be ambiguous [133]. In the past, ELSI approaches were often combined with efforts to inform the public about scientific developments [132]. Here, the idea was that by educating the public, they would become more in favour of scientific developments in general.

As a response to the shortcomings of ELSI, post-ELSI approaches to address the societal impact of new technologies have been introduced. One of these approaches is responsible research and innovation (RRI), a term that is widely used by the European Commission [134]. Rather than informing the public about scientific advancements, RRI is about engagement with society and other stakeholders, thereby developing innovations through co-production [135]. Compared to ELSI, RRI puts more emphasis on the research process than on the research outcomes [132]. Stilgoe et al. described four dimensions of RRI: inclusion, anticipation, reflexivity, and responsiveness [136]. Inclusion refers to the early engagement of a diverse set of stakeholders in the innovation process. Anticipation is about predicting outcomes of future research, among other things by a constant assessment of technologies and by involving the public. Reflexivity is related to both inclusion and anticipation, and aims to take organisational and societal values into account through the deliberation of variety of parties. Finally, responsiveness is about the constant adaptation of strategies as more knowledge about and views on certain innovations become available. Kwee et al. added two dimensions to the same four dimensions to cover all concepts described by RRI: openness & transparency and sustainability [137]. Here, openness & transparency refers to the availability of knowledge to everyone. Sustainability refers to both social and environmental sustainability, where social innovation is about the inclusion of societal values, whereas environmental innovation is about the efficient usage of resources.

Since SynCellEU has the long-term ambition to perform co-production in an innovation ecosystem, RRI is relevant to the case of this study. Related to RRI is responsible innovation (RI), a term that is more frequently used in academic contexts than RRI [135, 137]. However, Van de Poel et al. pointed out that RRI is especially used during the early stage of scientific developments and innovation [138], which is relevant to the case of SynCellEU. For this reason, and since SynCellEU uses the term RRI itself [114], we will use this term in this study, unless an article specifically refers to RI instead of RRI.

Although RRI has been discussed extensively in literature, it has been criticised for its lack in practical implementation [134]. Some even proclaim RRI to be a "buzzword", and although the fuzziness of buzzwords could promote the exchange of different perspectives [139], it complicates the question what it is that should be changed. Examples on the practical implementation of RRI have arisen over the last decade [140], but remain scarce [132]. This could possibly explain the increasing popularity of open science over RRI on the European political agenda [141]. Still, open science relates to the RRI dimension of openness & transparency identified by Kwee et al. [137]. Furthermore, scholars continue to point out the importance of early stakeholder engagement and involving the public, for example in the development of a synthetic cell [9]. This illustrates that, although the term RRI is losing momentum, its ideas remain present. For this reason, we will speak of "RRI elements" in this thesis, with which we mean aspects that relate to the six RRI dimensions as described by Kwee et al. [137].

7.2 Aim of the project and research questions

The aim of this research project is to find out how the foundation of an innovation ecosystem can be built. Due to the low technology readiness level (TRL) of synthetic cells, they are less attractive for industry at this early stage compared to more developed technologies. However, literature describes an increasing demand for early stakeholder engagement and mutual responsiveness, i.e. to include stakeholder groups like industry and society from the beginning of the innovation process [134, 137, 142]. To investigate how collaborations with these parties can already be formed, we will present a case study on SynCellEU in this study. This case study aims to address the following main research question:

In what way can SynCellEU lay the foundation for a sustainable innovation ecosystem?

To answer this main research question, we will discuss four sub-questions. The first sub-question is concerned with the vision of SynCellEU to form an innovation ecosystem and is formulated as follows:

1. What does the vision of SynCellEU to build an innovation ecosystem entail?

Whereas the first sub-question is mainly concerned with the perspective of SynCellEU itself, it is present in an environment that has certain innovative characteristics. These characteristics are important to address, since they describe challenges and opportunities for SynCellEU in its goal to build an innovation ecosystem. We will do this with the following sub-question:

2. What does the innovative context of SynCellEU look like?

In social sciences, models are frequently used to grasp reality through discussing, evaluating, and connecting theories [143]. Innovation processes can be described by technology-push and market-pull models (Subsection 7.1.2). It should be noted that an innovation process is not necessarily described by a single model. Since the type of innovation influences the tasks of stakeholders, it will be interesting to study what innovation model or models fit to the case of SynCellEU, and to learn what this means for its ambition to create an innovation ecosystem. Next to innovation models, there exists a variety of collaboration models related to innovation ecosystems. Applying one or multiple of these models will be useful to obtain more insights in the current collaborations of SynCellEU, and to explore how new collaborations can be formed. Finding a suitable collaboration model will also provide us with the vocabulary to describe the current and aspired collaborations of SynCellEU. Therefore, we will address the following sub-question:

3. What innovation and collaboration models best fit to the case of SynCellEU pursuing to build an innovation ecosystem?

One of the challenges of the case is that synthetic cells are ethically controversial: it is unknown to what extent people will consider a synthetic cell to be alive, and what people *can* and *will* do with the knowledge to be obtained from building a synthetic cell. Because of these ethical issues, governmental and societal support for SynCellEU might be impeded. The controversial aspect of the case, however, offers opportunities as well. RRI is concerned with the influence of new technologies on society and the environment (Subsection 7.1.3). The term RRI is losing momentum, but we observed a remaining interest in the aspects described by RRI (Subsection 7.1.3). The case study therefore allows for the investigation on how RRI elements can be put into practice in the field of synthetic biology, which we will do with the following sub-question:

4. In what way can RRI elements be implemented in the innovation ecosystem SynCellEU aims to build?

Here, we will discuss the following RRI elements: public engagement, open science & open innovation, and sustainability. In Section 12.1, we will verify that these RRI elements are relevant to the case.

7.3 Project structure

In Figure 7.6, an overview of the project structure is provided. Here, arrows indicate what chapters serve as input for other chapters. We used solid, dashed, and dotted arrows to be able to distinguish arrows that cross each other - the type of arrow does not say anything about the strength of a relationship. The project consists of four parts. In this chapter, we provided the **background** of the project. Next to this, we presented the aim and research questions of the project. The **methodology** describes how we will answer the four sub-questions, where the different methods used are displayed with differently coloured arrows (Figure 7.6, see Chapter 8 for a more elaborate description on the different methods). Each of the different sub-questions will be addressed in a separate chapter (Chapters 9, 10, 11, and 12). The answers to the four sub-questions are the **analysis** part of the project. The details of the innovation ecosystem that SynCellEU envisions are still unclear (Section 9.2), which was one of the motivations to perform a systematic literature review on this subject in Chapter 11. Based on the answers of the first three sub-questions, we will identify RRI elements that are relevant to the case in Section 12.1. In the systematic literature review, we will describe that an entrepreneurial culture is important for the development of an innovation ecosystem (Section 11.8), which is one of the reasons why we will explore the innovative context of the case in Chapter 10.

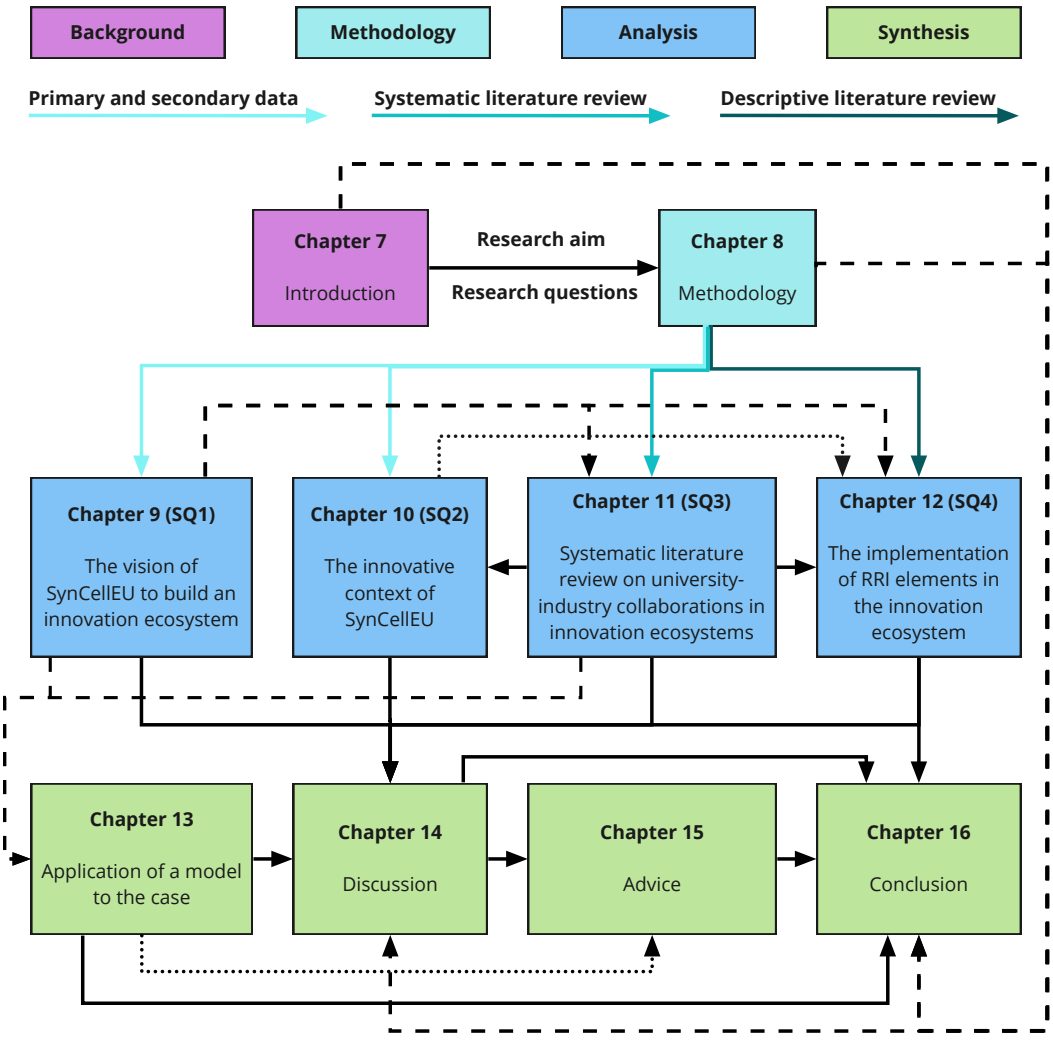


Figure 7.6 Structure of the project. The project consists of four parts: background, methodology, analysis, and synthesis. Arrows indicate what chapters serve as input for other chapters. Solid, dashed, and dotted lines are used to distinguish between arrows that cross each other. Figure made with Miro.

The answers to the four sub-questions will be combined in the **synthesis** part of the project. From the systematic literature review, we will select a model that can be applied to the case (Chapter 13). We will validate if the perspective of the selected model fits to the case of SynCellEU by relating it to insights obtained from sub-question 1. This is followed by a discussion in Chapter 14, where all previous chapters will be addressed. From the discussion, we will present an advice in Chapter 15. Part of the advice will be an intervention on the specification of synthetic cell applications. This intervention will be based on the model selected in Chapter 13. All chapters are summarised in the conclusion (Chapter 16). Finally, the two studies described in this thesis are integrated in Chapter 17 (not included in Figure 7.6). Each chapter, except the advice and the conclusion, will be summarised in a text box. The summary of this chapter is visible in Figure 7.7.

Summary of Chapter 7: Introduction

- BaSyC primarily performs fundamental research, whereas SynCellEU is also engaged in exploring synthetic cell applications.
- Innovation ecosystems have many different definitions. We adopted the definition of Granstrand and Holgersson [117] for its broad applicability and relevance to the case.
- RRI followed as a response to ELSI, and is currently increasingly replaced by open science. In this project, we will refer to “RRI elements” to describe the aspects fitting to the six RRI dimensions as described by Kwee et al. [134].
- The aim of this project is to find out how the foundation of a sustainable Synthetic Cell innovation ecosystem can be built.

Figure 7.7 Summary of Chapter 7: Introduction.

8
Methodology

In this study, we aim to answer the following main research question:

In what way can SynCellEU lay the foundation for a sustainable innovation ecosystem?

The main research question is addressed by combining the answers to the sub-questions into an advice. As part of the advice, we will propose an intervention in the form of a brainstorm session. The outcome of this brainstorm session could contribute to formulating the next steps required to build the innovation ecosystem.

8.1 The vision and context of SynCellEU

Sub-questions 1 and 2 will explore the vision and context of the case of SynCellEU, respectively:

1. What does the vision of SynCellEU to build an innovation ecosystem entail?

2. What does the innovative context of SynCellEU look like?

Sub-questions 1 and 2 are answered with both primary and secondary data. Primary data was obtained by performing semi-structured interviews, and secondary data was collected by studying web pages, online news articles and the Gravitation grant application for the BaSyC project. Semi-structured interviews were performed with the following experts:

- *Dr. Stefania Usai, Funding and Strategic Advisor at the Department of Bionanoscience (TU Delft), Programme Manager of BaSyC and SynCellEU (interviewed together with prof. dr. Marileen Dogterom)*
- *Prof. dr. Marileen Dogterom, Professor at the Department of Bionanoscience (TU Delft), Chair of the Steering Committee of BaSyC and Promotor of SynCellEU (interviewed together with dr. Stefania Usai)*
- *Prof. dr. Roel Bovenberg, Honorary professor Synthetic Biology and Cell Engineering at the University of Groningen, Senior Science Fellow at DSM Food Specialties, part of the International Advisory Board of BaSyC and also involved in SynCellEU*
- *Steven Lohle, MSc., former Business Relations Manager at the faculty of Applied Sciences (TU Delft)*
- *Dr. Denise Jacobs, current Business Cooperation Manager at the faculty of Applied Sciences (TU Delft)*

We interviewed these specific experts to gain insight from academia, industry, and professionals concerned with technology transfer, something that is an important part of innovation ecosystems (Chapter 11). We performed semi-structured interviews to be able to add interview questions based on the insights obtained during the interviews. An overview of the interview protocols used for the semi-structured interviews can be found in Appendix E. All semi-structured interviews were recorded with permission of the interviewees and fully transcribed using transcribing software provided by Wreally [144]. Interview recordings and transcripts were stored in a secured environment on Microsoft Teams. Moreover, the participants gave consent to use insights from the interviews for this study, and to mention their names. As described by Yin [145], we increased the validity of individual interviews by

identifying common patterns among them. Due to the length of the interviews with Usai, Dogterom, and Bovenberg, and the many topics they touched upon, these interviews were analysed with multiple rounds of coding. The first round was performed with provisional coding, which is a method where a list of expected themes is set up prior to the interviews [146]. The second round was done with structural coding, which assigns codes to text pieces of the transcripts based on themes that came up during the interviews [147]. An overview of the codes used for these interviews can be found in Appendix F. Coding was not found necessary for the interviews with Lohle and Jacobs, since these were mainly about the innovative context of the case, and relevant themes could be extracted by merely reading the interview transcripts.

8.2 Systematic literature review on university-industry collaborations in innovation ecosystems

In Section 7.2, we described the value of studying innovation and collaboration models in the context of a synthetic cell. Since innovation and collaboration are often combined in single models, the relationship between these models and the case of SynCellEU are studied in a single sub-question:

3. What innovation and collaboration models best fit to the case of the SynCellEU pursuing to build an innovation ecosystem?

8.2.1 Search terms

To answer sub-question 3, we performed a systematic literature review. Here, we studied innovation and collaboration models on the formation of innovation ecosystems. Since we found that innovation ecosystems often describe collaborations with a firm as central player without necessarily involving universities (Subsection 7.1.1), we ensured to select articles describing collaborations between academics and industry. The literature search was performed using Scopus, a renowned database developed by Elsevier including articles from more than 23,452 peer-reviewed journals [148]. We only searched for research articles, thereby excluding other pieces of text like reviews, conference papers and books. Below, the requirements for the systematic literature review are provided, as well as the search terms used to fulfil to the requirements:

1. Article describes innovation ecosystems
    - "innovation ecosystem" OR "innovation ecosystems"
2. Article involves academia
    - AND university OR universities OR science OR sciences OR academia OR academics
3. Articles involves a model
    - AND model OR models OR framework OR frameworks
4. Articles involves industry
    - AND industry OR industries OR company OR companies OR business OR businesses
5. Articles describes the formation of an innovation ecosystem
    - AND creation OR create OR creating OR emergence OR emerge OR emerging OR formation OR form OR forming OR "set up " OR "set-up " OR "setting up" OR establishment OR establishing OR development OR developing
6. Articles describes collaborations
    - AND collaboration OR collaborations OR network OR networks OR partnership OR partnerships

Articles were selected if the search terms were present in the title, abstract, or key words. The "AND" command ensured that a search term for each requirement was included, and the "OR" command ensured that only one search term per requirements was necessary for an article to be selected. By using the "OR" command, synonyms, plural forms and verb conjugations of search terms were included, thereby broadening the literature search.

8.2.2 Article selection

For the systematic literature review, we aimed to find articles describing innovation and collaboration models about innovation ecosystems. Since partnerships between academia and companies are an essential element of the case, we were only interested in articles where university-industry collaborations were discussed. The search terms described in Chapter 8 resulted in a total of 28 articles (Table 8.1). At the moment of performing the literature search, none of the articles were older than ten years, and 22 out of 28 articles were published within the last five years. Since the year of publication was not taken into account in the literature search, the recent reporting on innovation ecosystem models shows that it is an emerging field of research. The abstracts of the 28 articles were read to determine if the papers describe an innovation ecosystem model involving university-industry collaborations. If this was not clear from the abstract, the article was scanned to obtain more information.

After reading all abstracts, thirteen articles were excluded from the systematic literature review for multiple reasons. The most common argument was that the article did not elaborate on university-industry collaborations. In these cases, universities were often mentioned as a player of the innovation ecosystem, but no discussion on their role in the innovation ecosystem was provided. There were other reasons for not selecting an article as well, for example if the article was not available in English or Dutch. An overview of the excluded articles and the arguments for not selecting them can be found in Table 8.2.

From the literature search, twelve articles describing models on university-industry collaborations in innovation ecosystems were found (Table 8.3). Eight of these articles involved a case study, and together they described innovation ecosystems from a variety of different places in the world. The diversity in cultural and socio-economic contexts of the articles provided a comprehensive overview of the phenomenon of innovation ecosystems, where each article provided valuable insights and lessons that could be relevant for SynCellEU. Of the twelve articles, four were about the implementation of sustainability or responsible innovations in the innovation ecosystems. Due to their relatedness to RRI elements, the models described in these articles are discussed in Chapter 12, where sub-question 4 is addressed. However, some insights from these articles related to themes discussed in the systematic literature review are already discussed in Chapter 11. Next to the twelve articles describing innovation ecosystem models involving university-industry collaborations, three other articles were selected. These articles did not describe a specific model, but did elaborate on partnerships between universities and companies in innovation ecosystems, and were therefore still found to be valuable for the systematic literature review. An overview of the selected articles and the chapter in which they are discussed can be found in Table 8.3.

No.	Author(s)	Title	Year	Ref.
1	Schiuama & Carlucci	Managing strategic partnerships with universities in innovation ecosystems: A research agenda	2018	[149]
2	Ma et al.	The Impact of Local Government Policy on Innovation Ecosystem in Knowledge Resource Scarce Region: Case Study of Changzhou, China	2019	[150]
3	Xu et al.	Exploring innovation ecosystems across science, technology, and business: A case of 3D printing in China	2018	[151]
4	Shvetsova & Lee	Living labs in university-industry cooperation as a part of innovation ecosystem: Case study of South Korea	2021	[152]
5	Su et al.	A multi-platform collaboration innovation ecosystem: the case of China	2018	[153]
6	Hayashida et al.	Establishing of a base to build “the innovation ecosystem”	2015	[154]
7	Liu & Stephens	Exploring innovation ecosystem from the perspective of sustainability: Towards a conceptual framework	2019	[155]
8	Butler & Gibson	Research universities in the framework of regional innovation ecosystem: The case of Austin, Texas	2013	[156]
9	Levrouw et al.	Suspense as a driver for university-industry collaboration	2020	[157]
10	Nyamaka et al.	The components of an innovation ecosystem framework for Botswana’s mobile applications	2020	[158]
11	Silva & Ramos	Academic medical centers as innovation ecosystems: Evolution of industry partnership models beyond the Bayh–Dole act	2018	[159]
12	Jiang & Zheng	Coupling mechanism of green building industry innovation ecosystem based on blockchain smart city	2021	[160]
13	Lopes et al.	Regional innovation ecosystems and smart specialization: Opportunities and challenges for regions	2020	[161]
14	Sáez-Martínez et al.	The role of university in eco-entrepreneurship: Evidence from the eurobarometer survey on attitudes of european entrepreneurs towards eco-innovation	2014	[162]
15	Traitler et al.	Reinventing R&D in an Open Innovation Ecosystem	2011	[163]
16	Fischer et al.	Knowledge transfer for frugal innovation: where do entrepreneurial universities stand?	2021	[164]
17	Gunn	Multi-disciplined ecosystem-centric bioentrepreneurship education: Case study – University of San Francisco (USF)	2021	[165]
18	Kravchenko et al.	Research and business cooperation: International practice and Siberian experience	2019	[166]
19	Bandera & Thomas	The Role of Innovation Ecosystems and Social Capital in Startup Survival	2019	[167]
20	De Carvalho et al.	Institutions that foster innovative entrepreneurship in Brazil: Mapping and connections	2020	[168]
21	Joseph et al.	What corporates can do to help an innovation ecosystem thrive – and why they should do it	2021	[169]
22	Polónia & Gradim	Innovation and knowledge flows in healthcare ecosystems: The Portuguese case	2021	[170]
23	Liu et al.	An investigation on responsible innovation in the emerging shared bicycle industry: Case study of a Chinese firm	2019	[171]
24	Simba	A new model of knowledge and innovative capability development for small born-global bio-tech firms: Evidence from the east midlands, UK	2015	[172]
25	Shilpa & Bhattacharya	Bilateral S&T organisation as an innovation intermediary: Case study of Indo-French cell for water sciences	2020	[173]
26	Komninos & Tsarchopoulos	Toward Intelligent Thessaloniki: From an Agglomeration of Apps to Smart Districts	2013	[174]
27	Cresswell et al.	Accelerating innovation in health care: Insights from a qualitative inquiry into United Kingdom and United States innovation centers	2020	[175]
28	Sinell et al.	Uncovering transfer – a cross-national comparative analysis	2018	[176]

Table 8.1 Overview of the systematic literature review literature before article selection.

Reason for exclusion	Article number(s)
Proposal of personal vision on innovation ecosystems, from 2015 with zero citations	6
No English or Dutch version of article available	8
No elaboration on university-industry collaborations	10, 12, 20-22, and 24-27
The article is a review	13 and 15

Table 8.2 Overview of the articles excluded from the systematic literature review. Article numbers correspond to "No." in Table 8.1.

Reason for selection	Article numbers	(Model) discussed in
Describing a model on university-industry collaborations in innovation ecosystems	1-5, 9, 19, and 28	Chapter 11
Describing a model on university-industry collaborations in innovation ecosystem involving an RRI element	7, 14, 16, and 23	Chapter 12
Describing university-industry collaborations in innovation ecosystems	11, 17, and 18	Chapter 11

Table 8.3 Overview of the articles selected from the literature search and the chapters in which they are discussed. Article numbers correspond to "No." in Table 8.1.

To create an overview of the identified models, we developed a Model Classification Coordinate System (MCCS, Section 11.4). Here, models were classified based on their focus on innovation, collaboration, academica, and industry. Next to this, models were classified as either being a descriptive, strategic, or analytical model (see Section 11.4 for model type definitions). The MCCS was also deliberated to select a model that could be applied to the case of SynCellEU. Next to presenting the models identified by the systematic literature review, we explored differences and similarities between the descriptions of the articles. This was done by constructing a code-to-article matrix (Appendix G), where we indicated in what articles and to what extent specific themes and concepts were described. Subsequently, we identified overlapping themes and used the code-to-article matrix to elaborate on them. We also related the identified models and themes to the case of SynCellEU by referring back to the answers of sub-question 1 and 2. In this way, we both validated the interview results, and were able to find a suitable model to apply to the case.

8.3 The implementation of RRI elements in the innovation ecosystem

From Section 7.1.3, it followed that literature agrees on the fact that RRI elements should be effectuated, but that there is a lack of knowledge in how RRI elements should be put into practice. We address this issue with sub-question 4:

4. In what way can RRI elements be implemented in the innovation ecosystem SynCellEU aims to build?

Sub-question 4 is answered by identifying what RRI elements are relevant to the case. We did this by first extracting the following RRI elements from the systematic literature review: public engagement, open science & open innovation, and sustainability. We subsequently compared the extracted RRI elements with the six RRI dimensions described by Kwee et al. [137]. Finally, we verified the relevance of the RRI elements based on the interview transcripts, where we asked the interviewees how they are currently implementing RRI elements in their daily work and what challenges they experience in doing so. The identification thus mainly focused on what RRI elements are relevant in setting up collaborations between academia and industry for SynCellEU and the field of synthetic biology in general. Subsequently, a descriptive literature review on the implementation of these RRI elements was performed, where articles from the systematic literature review of sub-question 3 as well as other papers were included. A systematic literature review on the concept of RRI was beyond the scope of this study, since we only focused on specific RRI elements and their relationship with the case.

A summary of the methodology of this study is provided in Figure 8.1.

Summary of Chapter 8: Methodology

- We collected primary data (semi-structured interviews) and secondary data (web pages, online news articles, and the BaSyC grant application) to answer sub-questions 1 and 2, that focused on the vision and innovative context of SynCellEU.
- We performed a systematic literature review on models describing university-industry collaborations during the formation of innovation ecosystems.
- We developed a Model Classification Coordinate System (MCCS) to classify models based on their descriptions on innovation, collaboration, academica, and industry. Models were also categorised as either being strategic, descriptive, or analytical.
- Based on the answers to sub-questions 1-3, we identified RRI elements relevant to the case. We performed a descriptive literature review on the implementation of these RRI elements.

Figure 8.1 Summary of Chapter 8: Methodology.

9

The vision of SynCellEU to build an innovation ecosystem

In this chapter, we will discuss the vision of SynCellEU to form an innovation ecosystem. First, SynCellEU's website will be analysed, to get a general idea of the collaboration, and to study how the programme presents itself. The analysis will be followed by a description of the long-term vision of SynCellEU. Then, it will be described what efforts are currently made by SynCellEU to make this vision reality, and what challenges and uncertainties they face in this process.

9.1 Analysis of SynCellEU's website

On the homepage of SynCellEU's website, they state three main goals of their collaboration: to study biology with a bottom-up approach, to explore the potential applications of synthetic cell research, and to promote the discussion on the ethical and RRI aspects of a synthetic cell ([114], accessed on 9 July 2021). In a tab on the homepage where SynCellEU describes what their impact is, they name many potential applications of a synthetic cell: from "drugs that are able to target specific locations and tissues in the body", to "new, smart and environment-friendlier materials for high-tech industry" and "facilitation of sustainable production of safe and healthy food" [114]. There is also a tab dedicated to the benefits and opportunities for industry and society, where it is mentioned that "pharmaceuticals, food, nutrition, self-healing materials, bioplastics and sustainable fuels" are products that stem from synthetic cell related research, and that "the interest of companies will grow even more as the reality of a synthetic cell comes closer" [114]. Here, it is also described how methods and techniques developed during synthetic cell research could lead to the formation of spin-off companies in the future. A final tab is dedicated to the question why efforts to developing a synthetic cell should occur now. This question is answered by describing that Europe and the United States are at the forefront of a synthetic cell revolution, by saying that "our knowledge on how to build synthetic cells from their basic parts is now at a tipping point: bringing all this knowledge together will lead to revolutionary new technologies" [114]. Here, it is also mentioned that "close collaboration of synthetic cell scientists with governments and industry is essential", and it is estimated that "in the next 5 – 20 years this scientific field has the potential to take on global challenges in health, food and sustainability" [114].

On the homepage, SynCellEU also presents itself as a cutting-edge community [114]. This community is very accessible to new members: after filling in a form on their website, SynCellEU will contact you to put your profile on their page. All members of the SynCellEU community are thus presented on their website. An example of the profiles of the SynCellEU community is visible in Figure 9.1. It shows where people work, and community members can upload a profile picture and add keywords describing their interests. When clicking on a certain keyword, all people who chose this keyword are displayed, facilitating the process of finding people who work on specific subjects. Some community members have also written a small piece of text describing why they support SynCellEU, which becomes visible when hovering your cursor over their profile picture (depicted for the third person in Figure 9.1, where he shows his gratitude for being able to connect with amazing researchers).

Figure 9.1 Example of how the SynCellEU community is presented on the SynCellEU website. Community members can upload a profile picture, their institution is displayed, and they can add keywords describing their activities. People can also describe their reasoning for joining the SynCellEU community, which becomes visible when hovering the cursor over a profile picture. Figure retrieved from the SynCellEU website [114].

The majority of the community members visible on the website of SynCellEU are scientists affiliated to universities or research institutes. Most of these are situated in the countries where the founding organisations of SynCellEU are from: the Netherlands, Germany, the United Kingdom and France. However, there are also researchers involved from other countries in Europe, like Spain, Croatia and Finland, and a few researchers from other parts in the world like the Unites States and China. A bioinformatics student is also part of the community, showing that you do not have to be a prominent researcher for your profile to be put on SynCellEU's website. Next to researchers and students, people working at four different companies are also part of the community. These companies are of special interest for the case, where the formation of collaborations between academia and industry will be studied, and will be further discussed in Section 10.2.

A news slider highlighting scientific breakthroughs related to synthetic cell research is displayed on the SynCellEU homepage as well. A news article teaser that is part of this slider is visible in Figure 9.2. Next to describing the contents of the scientific findings, the news article teasers often show implications for potential applications. In Figure 9.2, it is for example shown how the research of ERC prize winners prof. dr. Anna Akhmanova and prof. dr. Marileen Dogterom could be used to "manipulate cells and treat diseases such as cancer" [114]. Another section of the slider describes how prof. dr. Jean-Christophe Baret developed a technique where cellular functions can be reconstituted using computer chips. Here, it is said that "in the coming years their research could lead to more sustainable solutions, for bioresources or waste management" [114]. A similar news slider is presented on the BaSyC website. However, the articles featured in the slider of BaSyC are more focused on the progression of building a synthetic cell, and less on the potential applications that this synthetic cell could have, indicating the different scope of the two collaborations.

Figure 9.2 News article teaser part of the slider on the SynCellEU website. The slider contents often describe potential applications of the research presented. Figure retrieved from the SynCellEU website [114].

Next to showing information about the initiative and the community, the website also has a page dedicated to events related to synthetic cell research. Here, both future and past events are displayed. The past events listed are three symposia organised by SynCellEU itself, from 2017 to 2019. In 2020 and 2021, events were not planned or cancelled, due to COVID-19 restrictions or other undetermined reasons. In 2022, the "International Conference on Engineering Synthetic Cells and Organelles" is planned in The Hague. Next to this, other (online) conferences organised by external organisations and related to synthetic cell research are presented as future events ([114], accessed on 27 February 2022).

9.2 The long-term vision of SynCellEU

We interviewed dr. Stefania Usai and prof. dr. Marileen Dogterom, Programme Manager and Promotor of SynCellEU, respectively. In their interview, they stated that they have the vision for SynCellEU to become an innovation ecosystem. Here, they have the goal to develop innovations together with companies through knowledge co-production. This innovation ecosystem could also become a public-private partnership (PPP) on the longer term, where there are strong collaborations between academia, industry and the government. The exact type of collaborations and how they will be formed, however, are still unclear due to many uncertainties. Usai: *"There's not one way to do this. It depends on the field of research, the current circumstances, on political and social developments, so it's very difficult to say: 'It's going to happen like this'. It's difficult to plan."*

Usai explained that they certainly want to form their collaborations on a European level, but that this increases the level of uncertainty: *"On a national level, we can act faster with BaSyC as a national consortium. We are more flexible and can achieve more, but we are limited by our country. Once we start to form collaborations abroad, there are developments in other countries we have to take into account. So it's a very complicated interplay between all kinds of factors, both national and international, and depending on the specific research field. There is no given recipe to go from here to there."* Usai also indicated that this is a process with a long duration: *"This is not something that will happen tomorrow."*

An example of a successful Dutch PPP is BE-Basic, a partnership working on bio-based solutions using industrial biotechnology [177]. Similar to BaSyC and SynCellEU, they started as a national consortium that was called B-Basic. This national consortium was later expanded with international collaborations in the BE-Basic programme. Dogterom explained why she thinks that you can and cannot compare SynCellEU with BE-Basic: *"BE-Basic is based on more applied fundamental research, so the link between the lab and the company is way shorter. For us, it is a whole other stage where we normally wouldn't even be in contact with industry. But because we foresee it will be interesting in the long term, we try to start this link early in the process."* Dogterom also indicated that she thinks that BE-Basic already had more existing collaborations between academia and industry. She explains that SynCellEU does not have these collaborations yet, but that they do have a vision where industry shows interest for.

I spoke to prof. dr. Roel Bovenberg in another interview. Next to being member of the International Advisory Board of BaSyC, he has been part of many European and American PPPs. Bovenberg: *"The beginning [of these PPPs] is often a bit fuzzy, not crystal clear. But with interested companies and PIs, for whom it often is a new adventure as well, [you start to work on the following questions:] How do you work together in a constructive way, and how do you build a team feeling within a consortium? Here, you should optimally benefit from the knowledge development and be capable of proceeding to knowledge transfer."* Bovenberg agrees with Usai that BaSyC is limited since it is a national programme, and that international collaboration is essential to really develop the synthetic cell field: *"Eventually, with all due respect, the Dutch initiative, despite the high quality of it, is small. (...) It is nice that there are initiatives in different European countries, like in Germany, and in France, and so on, but it is even nicer when you can find a European umbrella where different European countries work together to powerfully continue these research lines, which takes years. With, ideally, a good division of tasks and such."* He acknowledges that SynCellEU has the potential to become an international innovation ecosystem or PPP, but indicates that they should make concrete plans to achieve this.

9.3 Current efforts towards a coordinated European collaboration

It is thus still uncertain what the future holds for SynCellEU, but efforts towards a more international oriented consortium are made. Usai: *"You try to develop a vision: where do I want to be in five or ten years? What is the goal? Then you try to find the way towards that goal with the instruments that are present, both financially and organisationally. You also try to influence that the right instruments are made available."* One of the instruments that SynCellEU is currently lobbying for in Brussels is a European grant that promotes medium-scale coordinated projects. Currently, there does not exist such an instrument. Dogterom: *" (...) we don't get this further if there aren't any coordinated programmes with funding that belongs to that. No one can really make the next step, everything we do is dependent on whether there is funding to do it. Currently, the funding is scattered a lot. We can all keep our [research] groups running in all sorts of ways, but there is no coordination. In Brussels, we say that coordination is required over these individual initiatives if you really want to do something with [synthetic cells]."*

Dogterom explained that the European Research Council (ERC) grants are very useful forms of funding that can be used to perform a lot of research. They are, however, designed for individual or a small number of groups and the ideas that live there. As a result, there will be no coordination when applying for ERC grants. On the other hand, there has been the opportunity to apply for gigantic top-down programmes, like the European Future and Emerging Technologies (FET) Flagships. In the past, SynCellEU applied to become such a FET Flagship. Here, they made it to the second selection round, but then it became apparent that the formation of new FET Flagships was cancelled. Dogterom indicated that, especially for fundamental research, there is no suitable funding mechanism for projects that comprise more than a few research groups, but are smaller than the programmes for which the FET Flagship grants were intended. They therefore advocate for an instrument where research groups with a common goal can work together on a medium-sized project in a coordinated way. It is not necessary that these groups are assigned from Brussels, but there should be a coordinated theme where 10-15 groups can apply for, to ensure that the collaboration is not too small.

Bovenberg also indicated the importance of the lobbying activities in Brussels: *" One has to look beyond their own PI group, go to Brussels, approach companies and other stakeholders that could have an opinion about [SynCellEU] in one way or the other, or could use something from [SynCellEU]. One should approach them very actively and constantly involve them in the development. It sounds simple, but it is a major task, to get that done."* He also pointed out the importance of finding out what programmes there are, how to qualify for them, and how to bring attention to unknown subjects: *" (...) it is introducing yourself and putting yourself on the map, and at the same time finding out what the possibilities are, how it works, how to ensure that your field is sufficiently in the picture, and what to do to be successful at applications, especially when you want to talk about larger European programmes."*

Usai explained that the European lobby consists of multiple activities. On the one hand, they visit activities organised by the EU or other organisations, such as Knowledge4Innovation. These events are meant for European parliamentarians, other politicians and policy-makers to be informed about what is going on in the fields of science, technology and innovation. At these events, SynCellEU organises round table discussions, where people also give pitches about specific subjects. Usai mentioned that she experiences these round table discussions to be very useful: *"You for example hear what kind of issues are currently relevant for policy-makers."* On the other hand, SynCellEU invites people to their own events, which include symposia and lab tours. SynCellEU hopes to plant a seed and to create the "buzz" about synthetic cells at the events that they attend or organise themselves. Next to this, SynCellEU submits proposals for topics to the National Contact Point, which can be seen as the link between the European Commission and universities, professional associations, and companies in some cases. These topics are the issues that will be discussed in Brussels. Usai: *"Together with the other lobbying activities, we hope that [this helps to put our topics] on the agenda."*

SynCellEU thus aims to clarify the importance of a European collaboration aimed at building a synthetic cell. Multiple arguments that could be used in this lobbying process were mentioned by Bovenberg. Firstly, he pointed out that Europe has a good scientific basis for fundamental research, that is also

well supported and financed by the EU. He also indicated that, traditionally, Europe is more focused on the bottom-up approach of synthetic biology. Steven Lohle, former Business Relations Manager of Applied Sciences at TU Delft, explained that this is caused by a more present top-down approach in the Unites States, where there are less legal restrictions. Lohle also thinks that one has more profit from the bottom-up approach, since it offers more insights.

The proper bottom-up basis in Europe provides a starting point in forming a European collaboration. It even seems that Europe currently has the most initiatives on the bottom-up development of a synthetic cells compared to other parts in the world, like Asia and the Unites States. This means that Europe has a knowledge head start, which can be used as a second argument. There is interest in similar initiatives in other parts of the world, and Europe could lose its head start when it does not act. The third argument that could be used are the potential applications of synthetic cells and how they could contribute to society in the future. For this, it is important to already start thinking about applications, despite the difficulty to do this during the early phase of the project. Defining application fields and the challenges corresponding to that will be discussed in Chapter 10.

9.4 Formation of collaborations with companies

Despite the fact that there is not yet any funding available to form a coordinated European collaboration, and that it is still hard to determine the exact applications of synthetic cells, SynCellEU already attempts to form collaborations with companies. Dogterom explained that the current contacts with companies are formed both through pre-existing contacts and by actively reaching out to companies to form new connections. She explained that there are always some people from industry that monitor scientific developments and that try to keep in touch for the longer term. These people also speak to each other in networks and consortia. Dogterom: *"In that way, it starts to snowball. It is a bit word-of-mouth marketing, that's the way it goes, apparently, here as well."* Through this word-of-mouth marketing, SynCellEU came into contact with some new companies. Dogterom indicated that they also make use of their own network when looking for new partners, which among others includes people who switched from academics to industry.

Another way to create new contacts with companies is by actively approaching them. One way in which SynCellEU did this, for example, was by joining the chief technology officer (CTO) dinner organised by the valorisation centre of TU Delft, which can be seen as a lobbying activity for companies. For these dinners, that always have a specific theme, mainly regional companies are invited. The dinners are a sort of relationship management activity between TU Delft and these companies. One time, the theme of the dinner was the synthetic cell, and people from SynCellEU gave a presentation during this event. Here, companies that one does not necessarily associate with a synthetic cell also showed interest, for example Tata Steel. Dogterom: "*We didn't really expect this, but they also found this interesting, with interest in materials, for example. So this is another route, then it slowly catches fire. And then you hear nothing from them for a while, but this can come back after some time.*"

In the interview with dr. Denise Jacobs, the current Business Cooperation Manager of Applied Sciences at TU Delft, she explained that companies approach universities for collaborations as well. She previously worked as scientist and project manager at DSM, where she worked on a project under the flag of BE-Basic. At DSM, they developed the demo version of a new machine that was able to measure enzyme activity using calorimetry. Here, DSM reached out to Leiden University and TU Delft for academic support, and to validate if the approach offered an interesting technology to measure enzyme activity. She observes companies approaching the university in her current position as well: *"There are companies that contact the Innovation & Impact Centre, because it is an easy entrance to all sorts of institutes and business developments, and to gauge what is happening at universities, what is going on and where they can join."*

The formation of university-industry collaborations is thus an interplay, where both parties can take the initiative. However, as described in Section 9.2, it is likely that the research performed by BE-Basic was in general more applied than the current research of SynCellEU. Since the current level of synthetic cell related research performed by companies is probably limited, it is less likely that companies will

approach SynCellEU for collaborations at this stage.

Bovenberg made clear that "*you don't just form collaborations,*" and that this is something that requires time to develop. He indicated the importance of cultivating interest, which can be seen as the marketing and sales of your research. He also pointed out that it is important to invite people from the beginning, and to create a climate that encourages people to join discussions because of the interesting and exciting research. SynCellEU should not expect that collaborations are immediately formed, but they should arouse interest in the research they are doing. When, after some time, people become interested in certain research lines that can possibly be used for applications, SynCellEU should have an open attitude towards these opportunities. Bovenberg also mentioned that SynCellEU should not be afraid that knowledge or skills will leak in their efforts to form collaborations, since this can be solved with confidentiality agreements. To summarise, Bovenberg gave the following advice in forming collaborations: "*Get to know each other intensively, and then you will see that opportunities will emerge to get to concrete collaborations. That is by far the most powerful way to do it. [SynCellEU] has, in principle, the possibilities to start such an ecosystem, but you have to work hard for that.*"

Bovenberg mentioned that the majority of companies will probably be cautious and reserved to form collaborations, since they already have their own existing research lines and products. As a result, you have to be very good to fit in somewhere with something radically new. Being active in industry, Bovenberg also knows how challenging it is to form collaborations from the company side. He indicated that you never work alone, and that you have to convince other people about the value of forming certain collaborations. These people are not all scientists, but also people from management working in a business environment. It is often already challenging to convince people that it is useful and meaningful to set up contacts with for example a SynCellEU, let alone to really form an official collaboration. Next to this, he indicated that you do not get something for nothing, and that you should be able to motivate people to invest time and money in it. Here, he compared convincing your company to invest in a project with scientists convincing funding agencies to accept their proposals: "*There is a parallel world where you also have to deal with scarcity of people and money. Only when you have a very well substantiated story, you will get support within your company to focus on [a potential collaboration].*"

For every PPP where Bovenberg has been involved in, he has seen that they are preceded by a certain incubation time. During this incubation time, the programmes urgently asked companies what their thoughts are on certain issues, and what they should focus on. They also asked for more specific support, for example if a company can help them with a SWOT analysis, which is a method to identify the strengths, weaknesses, opportunities and threats of an organisation. Here, Bovenberg also pointed out the importance to genuinely incorporate the support or advice given. According to him, the Dutch collaboration BaSyC is currently in this incubation phase: "*That is also what I indicated in the advisory board, what kind of unique position they currently have, given the long duration of the Gravitation project, and the exciting subject, but that you, also in this phase, have to work hard if you eventually want to establish an innovation ecosystem and want to go to applications.*". Since the beginning of BaSyC, Bovenberg has passed on some company details to the management team, with whom BaSyC is currently having occasional conversations.

Future collaborations can either be promoted or prevented by competition. If synthetic cells start to become more relevant for industry, companies might experience a certain pressure to join the collaboration before their place is taken by a competitor. Since the research of SynCellEU is currently still in its fundamental step, Dogterom believes there is not yet any competition between companies in the field of synthetic cells: *"We are still in the pre-competitive phase. As long as there is nothing to get, the companies don't really make a point of it, until they start to sense something."* Bovenberg indicated that competition is typically relevant if companies are active in the same business. Finally, Bovenberg mentioned that consortia require clear agreements for good governance of all parties involved. This consortium agreement is typically poured into a legal framework, that allows industry employees to give constructive feedback that is mutually supported during consortium meetings. A consortium agreement can also allow competing companies to work together.

A summary of this chapter is provided in Figure 9.3.

Summary of Chapter 9: The vision of SynCellEU to build an innovation ecosystem

- SynCellEU presents itself as a “cutting-edge community”, and has the following three main goals:
    1. Study biology with a bottom-up approach
    2. Explore potential synthetic cell applications
    3. Promote ethical and RRI discussions on synthetic cells

- SynCellEU has the long-term vision to build a European innovation ecosystem, where synthetic cell applications are developed through co-production. The details of the envisioned innovation ecosystem are still unclear.

- SynCellEU lobbies for a European medium-sized instrument that coordiantes a collaboration between 10-15 research groups. The following arguments can be/are already used in this lobby:
    1. There is a strong basis for bottom-up biology in Europe
    2. Europe might lose its current head start to other parts in the world
    3. Synthetic cells have promising potential applications

- SynCellEU’s current connections are formed both through pre-existing contacts and by actively reaching out to companies.

- Due to the fundamental stage of synthetic cell research, it is less likely that companies will approach SynCellEU at this point, and that competition between companies will already play a role.

Figure 9.3 Summary of Chapter 9: The vision of SynCellEU to build an innovation ecosystem.

10

The innovative context of SynCellEU

This study aims to address in what way SynCellEU can build the foundation of a synthetic cell innovation ecosystem. In order to answer this question, it is important to explore what the innovative context of the case looks like. To this end, we will first discuss the different potential roles of a synthetic biologists, and what this means for innovation. Then, we will show the companies with whom SynCellEU already has existing contacts. After this, we will explore potentially interesting application fields and company types for synthetic cells. Finally, we will conclude with an analysis of the innovative environment at TU Delft, the university where the management of SynCellEU is situated.

10.1 The different roles of a synthetic biologist and expectation management

In their article on the implementation of RRI elements in a synthetic biology research centre, which will be further discussed in Chapter 12, Pansera et al. stated that in the United Kingdom, the role of the synthetic biologist has shifted from producing fundamental knowledge to contributing to the development of new applications over the last decade [132]. These increased efforts towards product commercialisation fit to the triple helix model described in Subsection 7.1.2, where universities, industry, and governmental organisations actively collaborate with each other. The shift in the activities can be related to the different roles of the synthetic biologist described by Schyfter and Calvert [178]. In their article, they define three different types of synthetic biologists: epistemics, pragmatic constructors, and committed engineers.

The first category, epistemics, describes the production of new knowledge as main task of synthetic biologists. Pragmatic constructors, on the other hand, are "those who see synthetic biology as new science to solve grand challenges or make specific products" [132]. Lastly, committed engineers are more focused on the technical aspect of synthetic biology. They believe that the field will allow researchers to understand biology in such a way that it can be used in a foreseeable and reliable way. According to Pansera et al, synthetic biology in the United Kingdom is highly influenced by the pragmatic constructors and committed engineers. Consequently, synthetic biology is framed as a field where other stakeholders expect impactful applications within a time frame that is not too long.

In the interview with Usai and Dogterom, we presented the described different categories, and asked them how they see the role of the synthetic biologist in the context of SynCellEU. Here, Dogterom pointed out that the case study by Pansera et al. is about cell engineering, where cells are already manipulated with a top-down approach to solve specific problems. Since SynCellEU aims to build a cell with a bottom-up approach, which is still in its infancy, Dogterom thinks that the researchers at SynCellEU mainly identify themselves as epistemics. However, she did indicate that the other two options are within the possibilities, and that this is also the direction that Brussels wants them to go. Dogterom does acknowledge that it makes sense that parliamentarians want to see concrete applications, but this does create a challenge in terms of expectation management. Dogterom: *" (...) before you know it, you are promising all kinds things from which you know that it will still take a very long time, but this is what people keep in mind. On the other hand, it is what works in Brussels. We can shout ten times that we will develop knowledge, that's nice, but what can we do with that?"*

This difficulty of managing expectations was also illustrated with an anecdote, where Dogterom and Usai spoke with a Member of the European Parliament: *" (...) she could then say very clearly that it all sounds good, but in the end she should be able to go on the streets and say something like 'I'm gonna*

solve the plastic soup', or 'I'm gonna solve cancer', so it should click in people's heads in one sentence to explain it, and at the same time, there shouldn't click something like 'this is dangerous, we cannot do this'. That also happens quickly. On the other side, I think you should remain authentic, without performing window dressing, even though people want you to do this."

Another pitfall mentioned by Dogterom is that in discussions about applications, you are often pushed towards a single innovation. *"It is not the case that we are doing this to solve one grand challenge or to make one product, we want to develop a technology that could play a role in many grand challenges in the future. So by saying in this discussion, 'I'm gonna solve disease X', or 'I'm going to make biofuel', you are throwing away a lot of potential. We therefore try to find a middle way in our leaflets, by concretely saying what [a synthetic cell] could bring, but by also stating that it is broad and that nothing will happen if we don't get the foundation stronger first."* Next to Dogterom, Bovenberg, also indicated that you should be very careful in presenting applications: *"You can make a lot of promises, but if this is not manifested and realised in the end, this is not a good thing. So you have to be cautious that you don't make promises that you cannot live up to in the end."*

10.2 Existing contacts with companies

SynCellEU already has contact with companies that have shown interest in their developments. A selection of these companies is visible in Figure 10.1. Here, it can be appreciated that they consist largely of chemical and food & nutrition companies, and some pharmaceutical companies. SynCellEU does not have any formal collaborations with companies yet, and no letters of intent have been signed. These companies have, however, indicated that they are interested in what is going on in the field of synthetic cells. Dogterom indicated that the current form of contact with industry is less interesting for smaller companies; they just want to do projects together. However, since the application of synthetic cells is an emerging field, small companies, such as start-ups, are perhaps more likely to make synthetic cell related products in the short to medium term compared to larger companies, who already have their existing core businesses. Lohle indeed mentioned that start-ups can play an important role in the delivery of equipment. He also saw this at the department of Quantum Nanoscience at TU Delft, where a whole new industry had to be built in the development of a quantum computer and quantum internet. To promote the formation of start-ups, and facilitate collaborations with them, an innovative and entrepreneurial culture is required, which will be discussed in Section 10.4.

Figure 10.1 Selection of companies where SynCellEU has contacts.

There are also some people working at companies that have joined the SynCellEU community that is described in Section 9.1. The largest of these companies is DNA Script, which is a company that has developed the first benchtop DNA printer working with an enzymatic synthesis technology [179]. DNA script is situated in France and in the United States, and has approximately one hundred employees. The three other companies that are part of the SynCellEU community are smaller ones where less than ten people work. Whereas a regular employee of DNA script is part of the SynCellEU community, for the three smaller companies it is the CEO that is part of the network. The first of these smaller companies is a start-up called Synovance. This start-up has developed a synthetic genetics platform,

that they mainly use to produce bio-based pigments, that can for example be used to produce safe and sustainable clothing [180]. One of the other smaller companies is Abvance Biotech, a start-up founded by scientists from the Centre for Biological Research in Spain. Abvance Biotech aims to develop therapeutics for immune system related diseases with an antibody based approach [181]. The last of the smaller companies is Yealthy, which is an "international organisation focused on fostering discoveries to enhance human potential" [182]. They do this by performing research, organising educational activities and by promoting innovation. Both Abvance Biotech and Yealthy state on their websites that they promote collaborations between academia and industry, making them interesting companies for the case study. DNA Script and Synovance, on the other hand, explicitly state on their website that they utilise synthetic biology in their production processes, making them interesting companies for the case as well. When comparing these companies to the ones displayed in Figure 10.1, it stands out that the ones described in this paragraph are in general more related to synthetic cell research. This could be explained by the fact that people working at these companies took the initiative themselves to join the SynCellEU community, potentially because they already foresee how it could benefit their company in the future.

Since SynCellEU currently does not have any formal collaborations with companies, they are not yet doing projects together. Their current activities primarily consist of having conversations and scanning if there are options for potential future collaborations. Dogterom indicated that it is noticeable that you often do not hear a lot about what companies are working on, but that they are able to globally explain why they are interested in the development of a synthetic cell. The companies are also updated on SynCellEU's activities through a news letter. On this mailing list, companies are also invited for workshops and meetings. One of these workshops involved a brainstorm session, where scientists, industry employees and other officials thought about potential applications of a synthetic cell in five, ten, or fifteen years (Section 10.3). An important point here was how these potential applications relate to existing technologies that can already be performed. One of these existing technologies is cell engineering, where the genetic code of cells is altered to achieve a desired outcome. From the brainstorm session, it followed that companies often think a new technology is only interesting when it cannot already be performed in a different way. Dogterom therefore indicated that they first have to move beyond this point before a synthetic cell will become really interesting for companies. Nevertheless, Usai mentioned that she observes a trend where companies want to jump in earlier in the process, and that they are also increasingly interested in alternative technologies when they are more sustainable than the technologies they are currently using.

Next to having conversations and brainstorm sessions with companies, some companies have also supported SynCellEU in funding applications. It was mentioned earlier that SynCellEU applied to become a FET Flagship (Section 9.3). In this application, people from industry supported them by writing a relatively non-committal support letter. These are partly people that switched from academics to industry, from whom it is believed that they are capable of estimating if a synthetic cell can be of importance for industry one day. In the end, they did not get this funding, but it did help them in mobilising a community. They can now show a list of companies and countries that were involved in this application to show how big they are. Some of these companies are also willing to support SynCellEU in their lobbying activities in Brussels, where they can indicate the importance of taking the research to the next level.

10.3 Synthetic cell application fields

In the previous section, we mentioned the brainstorm session organised by SynCellEU about potential future applications of a synthetic cell. The results of this brainstorm session are displayed in Figure 10.2. This infographic shows a timeline with predictions on scientific developments, as well as the technologies that are foreseen to stem from these findings. Part of the scientific developments correspond with the BaSyC work packages discussed in Chapter 1, such as "functional cellular modules" (WP1-3), "integrated cellular modules" (WP4), and "autonomously replicating cells" (WP5). On the timeline, it is expected that scientists are able to build autonomously replicating synthetic cells at the end of this decade.

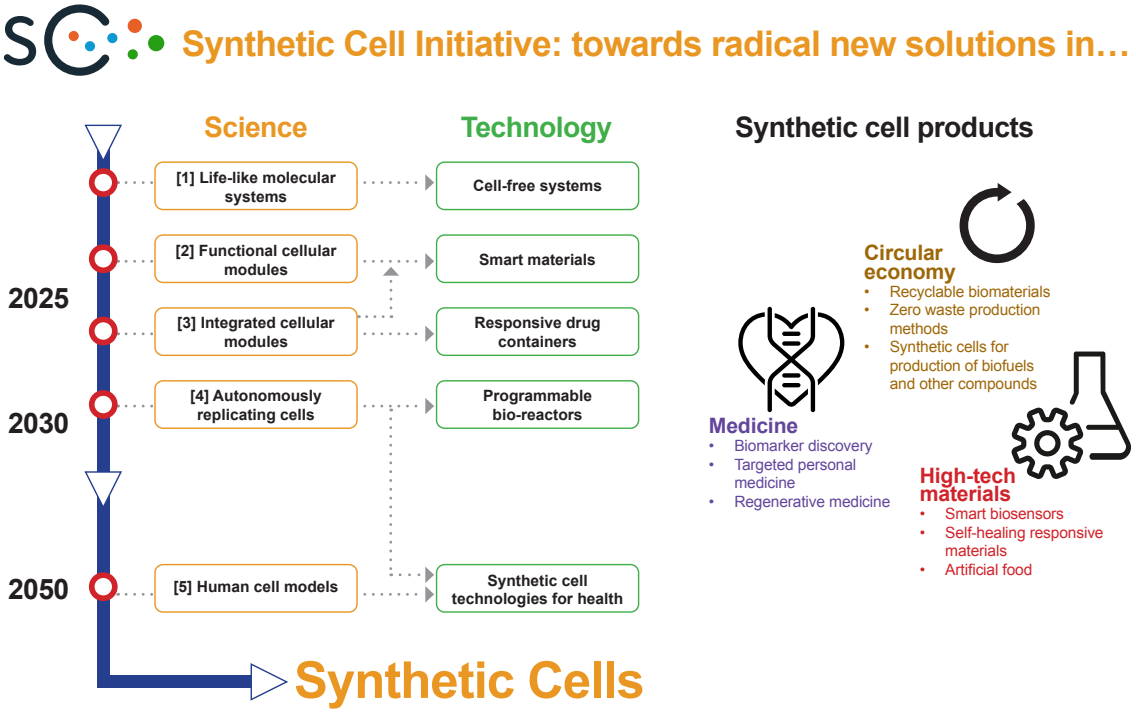


Figure 10.2 Results of the brainstorm session on potential future applications of a synthetic cell. Figure received from SynCellEU.

One of the technologies that synthetic cell research could contribute to are cell-free systems, an application that is already in use today [183]. This technology consists of *in vitro* systems to study biological processes. Instead of using a whole cell, cell-free systems only consist of specific cellular compartments to decrease complexity. It is expected that research conducted in this decade will contribute to the development of smart materials, vesicles for targeted drug delivery, and molecular reactors to control specific biochemical reactions. On the longer term, once synthetic cells have more similarities to actual human cells, it is expected that they will contribute to health technologies. From the technological developments that are foreseen, three specific product categories are defined: circular economy, medicine, and high-tech materials.

Through his active involvement in European and American PPPs, Bovenberg has a clear view on potentially interesting application fields for synthetic cells. During his interview, he elaborated on some of them. He thinks it makes most sense to start contacting companies working on molecular sensors, analysis software, and laboratory equipment. An already existing technique related to these fields is nanopore sequencing. With this technique, the movement of DNA through a nano-sized opening allows for the real time sequencing of the genetic code. In Bovenberg's eyes, this "piece of engineering" that combines biology, chemistry, physics, and geometry shows how molecular insights could be used to develop a sophisticated low-cost DNA sequencing technology. Currently, scientists are expanding this technique to analyse protein structures [184]. These techniques illustrate that it is not necessary to have a completely autonomous synthetic cell before applications can emerge, which is also pointed out in the infographic (Figure 10.2).

Bovenberg believes it is advantageous to start with the aforementioned application fields, since companies within these fields are used to work together with universities, and to perform the translation from the laboratory to practice. As Bovenberg put it: *"In the first place, it would make sense to develop synthetic cell applications in academic labs. Later, when it catches fire, it can be expanded to an increasingly broader spectrum of laboratories."* Synthetic cell practices could for example expand to medical research laboratories or companies that develop applications using cells. In case of the latter, industrial fermentation and cell breeding are options, but also the development of pharmaceutical pro-

teins. Bovenberg thinks that there will also be interest in the contribution of synthetic cells to cell-free systems, which corresponds to the results of the brainstorm session on future applications (Figure 10.2). Next to this, he thinks that synthetic cells could contribute to hybrid systems, where classical cellular production processes are modified. These processes are highly sensible, and Bovenberg believes that synthetic cells can possibly contribute to optimisations in this field of precision synthesis. Lastly, Bovenberg brings up a recent technological development: *"We are currently all in a pandemic. I'm not sure if this is fully just, but I also consider the mRNA vaccines as an application of synthetic cell research."* The attribution of mRNA vaccines to synthetic cell related research was also recognised by the scientific community [185].

According to Lohle, SynCellEU should base its innovation ecosystem on a single specific application field. He believes that, in order to get all parties required for a working innovation ecosystem, from multinationals to start-ups, it is essential to have a specific focus. As an example, Lohle mentions the convergence project of TU Delft and Erasmus Medical Centre. In this project, they aim to explore in what way technology can contribute to health solutions in the future. During an event of this collaboration, an engineer presented his work on removing the nucleus from a cell without breaking the cell wall. Here, the first response of one of the biomolecular scientists involved was the following question: *"Why would you do that?"*. The other scientist gave a typical engineering answer: *"Because we are able to"*. Subsequently, the biomolecular scientist figured that one can also put a nucleus back in a cell, and he started to wonder about the possibilities emerging from this finding. This is an interesting insight, but this only happens if people talk to each other within a certain framework, in this case about health exploration. In Lohle's opinion, building an innovation ecosystem based on a synthetic cell is too fundamental. He believes that astrophysics is the only field that succeeded in obtaining continuous funding for fundamental research, without the research necessarily having a direct impact on society. He thinks that this field achieved this through people's fascination for our place in the universe. The synthetic cell, however, comes too close to reality. Next to this, there are possible applications for synthetic cells.

Lohle does have a strong opinion on the application field that SynCellEU should focus on: *"One automatically goes into [health], but that is a minefield. You will end up in all sorts of religious discussions, while synthetic cells would be way more interesting for the world of materials. Then I'm talking about materials in its broadest sense. Materials are a fascinating world. Imagine you can grow a wall. That is far-fetched, but these are the concepts you will start to look at. That, instead of cutting a tree, you will literally grow a wall from wood, because you can grow the cells in that way. That was the direction in which we were thinking"*.

Lohle believes that, by focusing on materials, you do not exclude any applications, but you create a perspective that people can adhere to. In his words: *"In the end, everything is material. (...) It has less issues, but you don't exclude anything. You don't say 'I'm not going to do that'. The human heart, for example, also consists out of material. But if you say "materials", you create a different mindset. (...) I also think that "[building] a synthetic cell" has been a misconception to some degree, since it sounds like you are working on life. But the synthetic cell is not about life. It is about understanding life, but life is also built up from material."* According to Lohle, the current contact with companies like Shell and DSM fits to this view, since these are companies where logic and materials are highly important. Lohle's perspective is similar to one of the synthetic cell product categories identified by SynCellEU, namely that of high-tech materials (Figure 10.2). Other applications are not excluded with this idea, but they should stem from a motivation to produce new materials.

10.4 The innovative environment at TU Delft and beyond

Next to the existing contacts of SynCellEU, it is interesting to study its innovative environment to identify resources its can use to expand its network. In this study, we will mainly investigate the innovative context of TU Delft, the university where the management of SynCellEU is situated, and where half of the original steering committee of BaSyC is currently situated. For future research, it would be interesting to study the innovative environments of the other universities and research institutes affiliated to SynCellEU as well.

At TU Delft, the main organisation responsible for technology transfer activities and fostering innovations is the Innovation & Impact (I&I) Centre (previously the Valorisation Centre). Next to the university's tasks to offer education and to perform research, knowledge valorisation is performed by the I&I centre as the third mission of the university. On their website, it is stated that they promote collaborations between the university, industry, governmental organisations, and knowledge institutes based on their mission "Impact for a better society" [186]. They do so through four main types of collaborations or activities: PPPs, Corporate Innovation, Patents, and the Internship Offices and Career Centre. Next to BE-Basic, discussed in Section 9.2, TU Delft is active in eleven other PPPs, among others focused on photon therapy, quantum computing, and water innovations. Corporate Innovation offers companies the possibility to participate in collaborations with the university, and assists scientists in obtaining funding. Patents assists researchers in commercialising their findings and to protect intellectual property rights. Finally, the Internships Offices and Career Centre matches students with companies for internships, and provides career training to prepare students and recent graduates for the job market.

Within their role to promote collaborations between the university and the industrial world, the I&I Centre has conversations with SynCellEU to assist them in their goal to build an innovation ecosystem. Lohle held his position as Business Relations Manager from 2016 until 2021, thereby having experienced the start of both BaSyC and SynCellEU. In his time working at TU Delft, Lohle has seen a lot of changes in its valorisation procedures. He mentioned that in 2014, TU Delft was already working on so called "corporate accounts". These collaborations with companies like Shell, Philips, and ASML, were mainly maintained on a local level. Although TU Delft already was a precursor at that time, the collaborations have become a lot more professional since then. Lohle explained that they increasingly took the wishes of society into account: *"We started to look at the activities of the outside world, and how you can ensure that our scientists, who are all researching beautiful and fun subjects, can make choices in that palette of possibilities"*. Also for fundamental research, they started to investigate how this can be pushed towards societal issues.

The biggest turn they made, according to Lohle, is to start thinking about valorisation before the research is performed instead of when it has already been done, and how researchers and their passions can be framed within societal developments. Here, scientists can still perform fundamental research, but it should be clear what societal impact this research can have on the long term. A successful example of this are the quantum applications that were already mentioned in Section 10.2. These are specific applications, but at the same time, the scientists at this research department continue to perform fundamental research. Lohle also thinks that it is important to let the outside world show the research that scientists are doing - not necessarily because society will give input to fundamental research, but because this will give them insights in what is going on in the field. Next to identifying societal challenges and supervising scientists to fit their research to it, Lohle believes there is little that the I&I Centre can do for this highly fundamental research: *"In the end, it is the scientist who understands it at heart"*.

During her time at DSM, Jacobs participated in multiple university-industry collaborations, for example in the BE-Basic project (Section 9.4). Having an industrial background, she has some advantages in fostering university-industry collaborations in her current position as Business Relations Manager: *"You know how people work [in industry], how decisions are made, and what is important. That's what you take that with you. You can also take that with you in translating scientific research to practice. You can add your piece there. And thinking in [key performance indicators], business targets, and business cases. That really helps when you want to start collaborations."*

Jacobs only started in her function two months prior to the interview. Therefore, she could not yet give a total picture of the I&I Centre's role in facilitating university-industry collaborations. However, she clearly named different stages in which she can play a facilitating role: *"I have identified a few phases in that process myself, like initiating contacts and ideas, developing contacts and ideas, forming agreements, making the contracts of the agreements, and executing and evaluating them. In those different phases, I can supervise the process."* She indicated that she never does this alone, and similar to her predecessor, she mentioned that the scientist is in the lead. Next to her contacts with companies and

scientists, she can switch to other people of the I&I Centre that can add their part. Jacobs: *"There are many people involved in that. It's very diverse."* To attract companies to fundamental research, Jacobs believes it is important to gauge interest and to develop a story that fits to the outside world. Here, it is valuable to have discussion partners to develop that story. The greatest challenge that Jacobs foresees in facilitating university-industry collaborations is for the two parties to find each other. Jacobs: *"For SynCellEU, that's very important. There are a few big players that support the idea, but they don't contribute concretely in cash or kind. It's exciting how that will develop, and interesting to experience and to see that growing. (...) It's an organic whole. Sometimes things arise that you don't expect, sometimes things extinguish that you neither expect. You really have to steer on chances, and to keep people interested once they are interested. To shape the next phases, it helps to make agreements about when you come back or not."*

Next to the I&I Centre, other organisations focused on innovation and more specifically on entrepreneurship are situated at the TU Delft campus. One of these organisation is Delft Enterprises, who offers support to TU Delft students and researchers who want to turn their scientific findings into a spin-off company. They do this by focusing on "deep tech", which are "radical innovations that can solve major societal challenges" [187]. Entrepreneurship is promoted by offering advice, funding, and by connecting TU Delft students and employees to a "broad network of investors and experts" [187]. Delft Enterprises is a for-profit organisation connected to the I&I Centre. Lohle indicated that TU Delft Holdings (the parent company of Delft enterprises) is owner of all shares of Delft Enterprises, and that TU Delft owns all shares of TU Delft Holdings. Consequently, the main goal of Delft Enterprises is not to maximise profits, but to contribute to maximal societal impact from the knowledge generated at TU Delft.

Another organisation housed at TU Delft is YES!Delft, which is a business incubator set up in collaboration between the municipality of Delft and the university. Through their strong links with industry, YES!Delft is a clear example of a collaboration described by the triple helix model (Subsection 7.1.2). Over the last couple of years, YES!Delft has grown to be one of the largest incubators in Europe by realising the foundation of over 200 start-ups [186]. It offers physical spaces for start-ups to develop, mentorship possibilities, and network expansion options.

Despite the fact that there are technology transfer possibilities, Bovenberg believes that this is less internalised in Europe. According to him, Europe is quite good in the lower TRLs from 1-3 (Figure 10.3), where fundamental research is performed, but less active in the TRLs from 4-6, where there is a transition phase from scientific output to potential applications. Bovenberg believes that European programmes, as discussed in Section 9.3, should also focus on these intermediate TRL levels. In his words: *"This means that, next to the fundamental insights and research, you should also pioneer with possible application directions. This will allow you to have conversations with companies that are currently active in these fields, to see if they are interested and if they even know about the research"*. In this way, scientists can gauge if their research is already interesting for other stakeholders, or if it is still too fundamental.

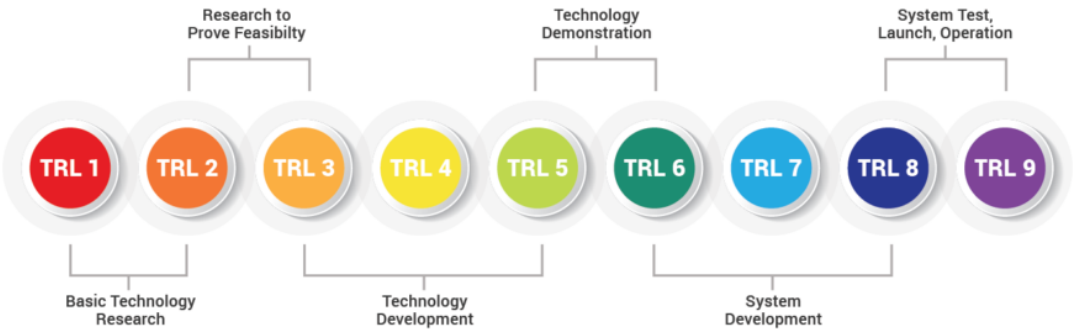


Figure 10.3 Overview of technology readiness levels (TRLs) and their characteristics. Figure retrieved from Biominas Brasil [188].

Bovenberg explained that in the Netherlands, it is less common to form a start-up. He thinks this is due to a somewhat less-developed entrepreneurial culture. He mentioned that in other countries, PPPs often lead to the formation of start-ups, where PhD students or postdocs start a company based on their own or a colleague's research. In the United States, for example, companies like Amyris, Zymergen, and Ginkgo Bioworks stem from PPPs focusing on cell engineering. Ten to fifteen years ago, these companies were formed as start-ups. Currently, they are large companies where hundreds of people work, and with investments of hundred million to billion dollars. Bovenberg: *"You should have some guts and dare to pioneer"*. Next to this, Bovenberg believes that it is important that the right resources are available to form a start-up, and that it should be supported by the principal investigator.

In an article on technology transfer, that will be discussed in the systematic literature review (Chapter 11), Sinell et al. suggest that transfer activities can possibly be promoted by career benefits, for example in the form of promotions, increased salaries, or patent rights. Lohle explained that such career benefits are not offered by the I&I Centre, but that support is offered based on the ideas that inventors have with their (potential) patent. Support can for example be offered in the formation of a start-up, or in the application for a grant. Next to this, it is regulated at TU Delft that an inventor receives one third of the net gain that a patent generates.

A summary of this chapter is provided in Figure 10.4.

Summary of Chapter 10: The innovative context of SynCellEU

- SynCellEU researchers currently see themselves mainly as producers of fundamental knowledge. They increasingly focus on applications, where expectation management is a big challenge.
- SynCellEU has no formal collaborations yet, but has contacts with companies that are interested in the development of a synthetic cell.
- SynCellEU can be supported in its funding applications by company contacts.
- Three synthetic cell applications fields were identified in an earlier brainstorm session: circular economy, medicine, and high-tech materials. These application fields are generally recognised by the interviewed experts.
- The innovative environment of TU Delft comprises of three important organisations:
    1. The Innovation & Impact Centre (responsible for technology transfer)
    2. Delft Enterprises (company that offers support for spin-off formation)
    3. YES!Delft (one of the largest business incubators in Europe)
- Europe is less active in intermediate TRL levels (4-6), which is attributed to a somewhat less-developed entrepreneurial culture.

Figure 10.4 Summary of Chapter 10: The innovative context of SynCellEU.

11

Systematic literature review on university-industry collaborations in innovation ecosystems

11.1 Unexplored avenues of innovation ecosystems

Next to finding a suitable model that can be applied to the case of SynCellEU, the systematic literature review will provide us with an overview of recent findings in the innovation ecosystems field. An overview of overlapping themes and concepts among the selected articles can be found in the code-to-article matrix (Appendix G). The article numbers displayed in this chapter can be found in Table 8.1. One common theme is that the concept of innovation ecosystems is not fully explored. In fact, 80% of the articles suggested this in some way (all articles except 1, 11, and 18). Su et al., for example, described that individual components of innovation ecosystems have been studied extensively, but that the connections between these parts and their functions are less defined [153]. Similarly, Xu et al. mentioned that it remains elusive how interactions between the science-technology and business ecosystems have an effect on the innovation ecosystem [151] (Subsection 11.5.3). Levrouw et al. described that arguments for companies to join partnerships are mainly lacking in literature [157]. Next to this, Ma et al. identified the literature gap on policies and their effect on innovation ecosystems, especially in regions where innovative capacities were lacking before [150]. The relationship between small and medium-sized enterprises (SMEs) and universities in innovation ecosystems was addressed as well [162]. At last, multiple articles (7, 16, and 23) stated that the implementation of specific RRI elements into innovation ecosystems is underexplored, which we will discuss in Chapter 12.

11.2 Relationship with innovation and collaboration models

From the analysis, we found that six out of fifteen selected articles related innovation practices in innovation ecosystems to the linear and/or non-linear innovation models (Subsection 7.1.2). Here, five articles specifically mentioned that the non-linear model applies to innovation ecosystems (1-4 and 7), and one other article (14) described how the linear innovation model has been extended by involving the public, thereby gaining characteristics of the market-pull innovation model. Shvetsova and Lee explained how the transition from test beds to living laboratories in South Korea changed the innovation process from a linear to a non-linear approach [152] (Subsection 11.5.5). On their article on the implementation of sustainability in ecosystems, Liu and Stephens described how innovation has evolved from a linear to a non-linear quadruple helix model [155].

The non-linear dynamics of innovation ecosystems were more frequently related to the helix framework. In fact, nine out of fifteen articles related innovation ecosystems, or collaborations that are common within innovation ecosystems, to the triple and/or quadruple helix models (1-4, 7, 9, 18, 23, and 28). These models, that were discussed in Subsection 7.1.2, describe collaborations between academia, industry and the government, where the public is added in the quadruple helix model. Kravchenko et al., for example, explained how university-industry collaborations are often described through conceptual structuring, of which the triple helix model is an example [166].

The articles also provided practical examples of collaborations described by the helix framework. In the article by Liu et al., it is described how data sharing by citizens allows the shared bicycle industry in China to predict peak hours [171]. The shared bicycle industry, that already had partnerships with universities and governmental organisations, extended its collaboration landscape from a triple to a quadruple helix model in this way. Another example of a partial practical implementation of the quadruple helix model is given by Sinell et al. [176]. In their article, they have studied multiple technology transfer offices (TTOs) across different countries. In some of these TTOs, bi-directional collaborations between science and industry were identified. Consequently, "transfer process can no longer be understood simply in terms of uni-directional "pulls" and "pushes"" [176], relating to the technology-push and market-pull innovation models (Subsection 7.1.2). The authors specifically mentioned that this is only a partial implementation of the quadruple helix model, since in theory, the model describes bi-lateral collaborations between all parties involved. The findings therefore illustrate that innovation ecosystems are often related to the helix framework, but that practical examples of full implementations remain scarce.

11.3 The dynamic character of innovation ecosystems

Except for one article (14), the dynamic character of innovation ecosystems was brought up by all papers. According to Kravchenko et al. [166], literature has extensively discussed the rapidly changing environments of innovation ecosystems, and how stakeholders should form sustainable collaborations within these environments to create competitive advantage. Similarly, Shvetsova and Lee described how the national South Korean innovation ecosystem constantly has to respond to a dynamically changing world to remain competitive [152]. These dynamics are for example illustrated by the rapidly changing preferences of customers. Gunn pointed out that for bioenterprises to be successful, they have to be resilient to the dynamic circumstances to which they are exposed [165]. These circumstances comprise, among other things, adaptations in the law, the activities of competitors, and potentially negative coverage in the media. Finally, Liu et al. stated that through interactions with society and the environment, the shared bicycle industry in China has created a more dynamic innovation ecosystem themselves [171]. These dynamic changes relate to the external stimuli Usai mentioned in her interview, to which SynCellEU constantly has to adapt its strategy (Section 9.2).

11.4 Model classification coordinate system

Before we will further discuss overlapping themes and concepts between the selected articles, we will first discuss the identified models. While performing the systematic literature review, it was found that articles often do not describe models that are purely about innovation or collaboration. Instead, the majority of the models is concerned with both innovation and collaboration, since these two activities are heavily intertwined. Although innovation as well as collaboration aspects are described in most articles, the models often have a stronger focus on one of the two concepts. Two other entities that come back in each article are academia and industry, since the systematic literature review selected for articles describing university-industry collaborations in innovation ecosystems. Here, articles often have a stronger focus on one of the two stakeholders as well. To create an overview of the different articles discussed in the systematic literature review, we developed the Model Classification Coordinate System (MCCS, Figure 11.1). The models discussed in this chapter are placed on the coordinate system, where their positions are based on their perspectives (more towards innovation or collaboration, and more towards academia or industry). Here, the placement was based on our own assessment after reading the articles. Next to the classification of their perspectives, the models are also categorised as either being a descriptive, a strategic or an analytical model.

Descriptive models illustrate the development of innovation ecosystems, or important actors within the ecosystem, either factual or fictional. Strategic models, on the other hand, make suggestions on the development of innovation ecosystems, either in terms of proposed research or practices to develop the ecosystem. Finally, analytical models involve statistical methods that assess innovation ecosystem performance. Next to creating an overview, the MCCS will be used to determine what model or models best fit to the case of SynCellEU.

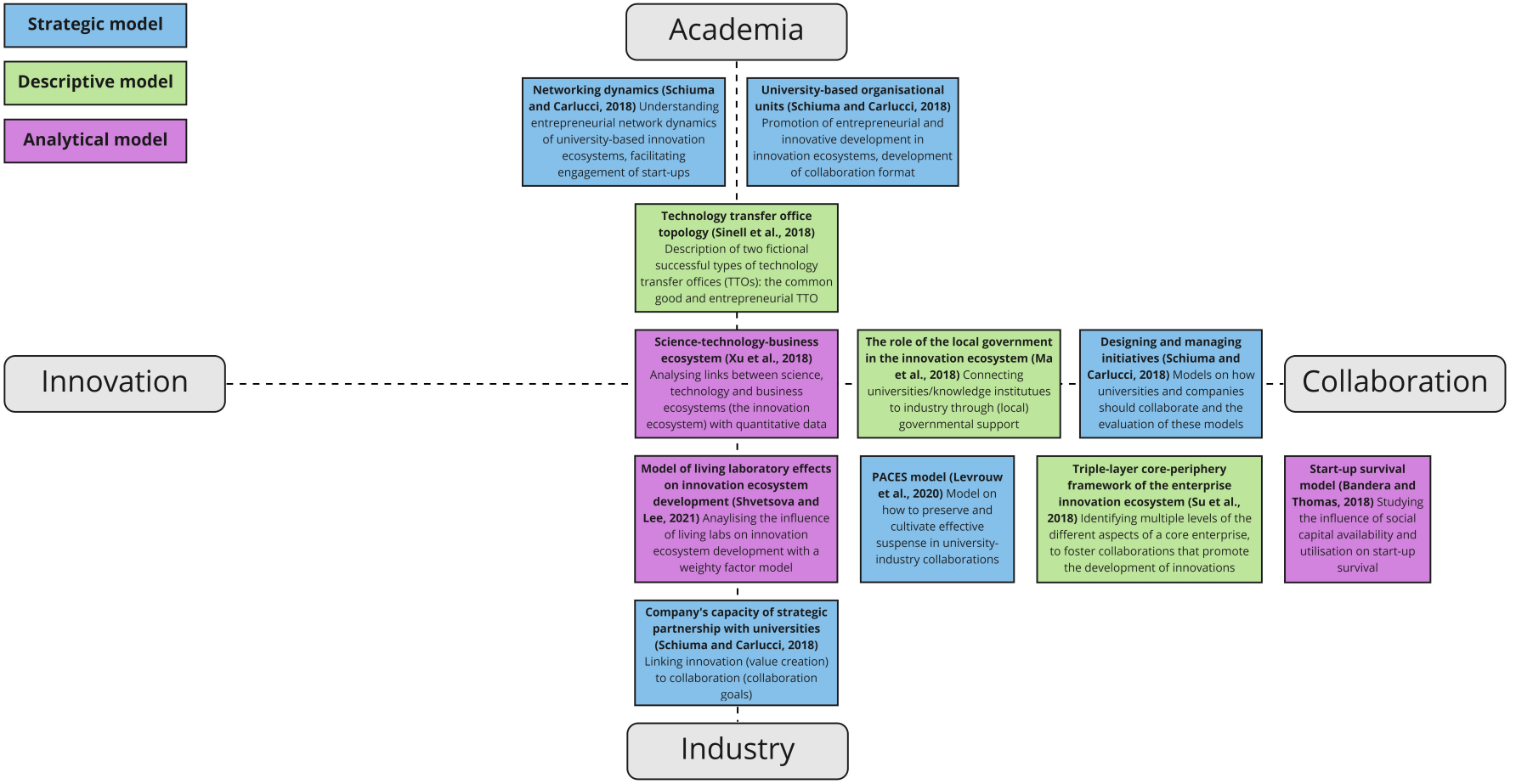


Figure 11.1 Model Classification Coordinate System (MCCS) to categorise and identify the perspectives of the models described in the systematic literature review. The position of the models indicates their focus towards academia or industry, and towards innovation or collaboration. The majority of the models are displayed on an axis, meaning that they provide a link between the entities of the relevant axis. Figure made with Miro.

When looking at the MCCS, we can appreciate that the majority of models are positioned on an axis. This means that the model provides a link between academia and industry, or between innovation and collaboration, depending on the axis where it is displayed. Only one model element is focused more on innovation than on collaboration, showing the relevance of forming partnerships in innovation ecosystems. The majority of models, however, involve both innovation and collaboration. Next to this, the number of models that are more focused on industry is larger than the amount of models with an academic perspective. This might limit the number of models that are applicable to the case of SynCellEU, but could also provide us with valuable insights about industrial perspectives on innovation ecosystems. Finally, there is a proper division of model types among the articles read: we found five strategic, three descriptive, and three analytical models or model elements. This means that we will study innovation ecosystems with different approaches. Except for the first model, that consists of multiple elements put at different positions on the MCCS, the models will be discussed starting with the ones focusing mainly on academia, and finishing with the ones mainly having an industrial perspective.

11.5 Overview of the investigated models

In this section, the identified models not specifically focusing on RRI elements will be described. Their placement on the MCCS will be discussed, and the relationship between the model and the case of SynCellEU will be discussed at the end of each sub-section. The identified models focusing on RRI elements will be discussed in Chapter 12.

11.5.1 Managing strategic partnerships between academia and industry in innovation ecosystems

The model developed by Schiuma and Carlucci consists of a research agenda to manage "strategic partnerships with universities in innovation ecosystems" [149]. Schiuma and Carlucci described that universities catalyse the formation of local innovation ecosystems. They mentioned Silicon Valley (San Francisco Bay Area, USA), Kendall Square of MIT (Cambridge, USA), and Block 71 (Singapore) as examples of highly successful innovation ecosystems where universities played an essential role in the development of the region. This was linked to the trend of open innovation, where universities increasingly exchange knowledge with companies to accelerate the development of novel applications. The authors were specifically interested in these successful cases, since they believe that strong university-industry collaborations can bring more prosperity to less-developed areas. They mentioned the region of southern Italy as an example, where, according to the authors, companies “lack an inner capacity for innovation and have weak ties with research institutions” [149].

Although the benefits of strong university-industry collaborations are increasingly recognised, the ways in which to form, maintain and evaluate these partnerships remain an active subject of study. The defined research agenda identified four perspectives that require a better understanding, to create stronger university-industry collaborations in the future (Figure 11.2). Since these perspectives cover different subjects, they will be placed individually on the MCCS. The first of these perspectives is "Networking dynamics", that is concerned with understanding the entrepreneurial network dynamics of innovation ecosystems where universities play a central role. It is about the multi-actor network that is formed around these universities, and activities that make universities more innovative. The aim to make a university more entrepreneurial to improve the innovation ecosystem requires a strategic planning. A question that was asked here, is how universities can facilitate interactions between start-ups and the innovation ecosystem. With this, the first perspective provides a link between innovation (what activities promote entrepreneurship?) and collaboration (how does this lead to collaborations with start-ups?), directed from universities (Figure 11.1).

The second perspective is called "University-based organisational units". It is related to the first one, but it is more concerned with the practical implementation of university-industry collaborations. It is about the organisational structures, functions and activities that are required to involve universities in the innovation ecosystem, which depends on the stage of innovation. It is also about the question how universities and companies should collaborate in practice, which depends on the goals of the partnership. With the second perspective, universities provide a link between innovation and collaboration as well, somewhat more focused on collaboration compared to the first perspective (Figure 11.1).

The third perspective, "Company's capacity of strategic partnership with universities", aims to study what factors influence the ability of companies to form successful collaborations with academia. This requires the development of an innovation value map, through which reasons for companies to collaborate with academia can be identified. This perspective provides a link between innovation and collaboration as well, but this time directed from industry (Figure 11.1). The fourth and last perspective, "Designing & managing initiatives", is about models on how universities and companies should collaborate in innovation ecosystems, indicating the relevance of this systematic literature review. It also deals with the evaluation of these partnerships, which requires both qualitative and quantitative indicators. This perspective is about collaboration between academia and industry, hence its placement in the middle right of the MCCS (Figure 11.1). The research agenda developed by the authors makes suggestions on areas of study that require further investigation. Therefore, its four perspectives are classified as strategic model elements.

Figure 11.2 Research agenda to manage strategic partnerships between academia and industry in innovation ecosystems. Figure retrieved from Schiuma and Carlucci [149].

Schiuma and Carlucci suggested to test the model in a "Contamination Lab" (CLab). CLabs are university organisations that are concerned with both (entrepreneurial) education activities and the promotion of innovation collaborations, for example by forming start-ups, idea labs and innovation consortia. Similar to three out of four perspectives of the research agenda (Figure 11.2), CLabs provide a link between innovation and collaboration. The authors therefore believe that CLabs are an interesting place to study the four perspectives identified in the research agenda. Next to this, they propose that CLabs can shift "ad hoc" university-industry collaborations towards "strategic partnership partnerships" [149]. Collaborations formed with the ad hoc approach are based on personal relatedness. Therefore, they are more formed on the basis of individual familiarity instead of similarity between the organisations. According to Frølund et al., the ad hoc approach typically leads to many collaborations, but with a low level of synergy [189]. Strategic ecosystem partnerships, on the other hand, require an investment of the university management in the collaboration, which could lead to more effective and sustainable partnerships.

In Section 9.4, we described that the current collaborations of SynCellEU partly arose from pre-existing contacts. Since these relationships are often on a personal basis, they could be identified as ad hoc collaborations. The development of a synthetic cell innovation ecosystem could therefore be promoted when SynCellEU shifts its collaboration strategy from a partial ad hoc approach towards more strategic ecosystem partnerships.

11.5.2 Technology transfer office topology

Next to two model elements of the research agenda described in the previous section, there is one other model that was mainly developed from an academic perspective. This descriptive model, developed by Sinell et al., discusses the position of technology transfer offices (TTOs) in innovation ecosystems. It is recognised by the article that in order to realise effective collaborations between universities, companies, governmental organisations, and society, strong knowledge and technology transfer (KTT) is required. TTOs are an increasingly important actor in this environment, where they adopt the role of a mediator between science, technology, policy, and citizens.

Due to the large amount of actors that TTOs interact with, there is a great variation in forms of transfer between different offices. Some of these forms can be expressed in numbers, like the amount of patents or spin-offs in which a TTO played a facilitating role. Other forms, like promoting university-industry collaborations or giving advice about transfer activities, are more difficult to grasp. Since effective KTT is essential to build a thriving innovation ecosystem, assessing TTO performance can be useful. The authors therefore studied TTO practices of renowned research institutes from five different countries: Germany, Sweden, Switzerland, Italy, and Israel. For this, they interviewed 34 senior KTT managers from these five TTOs. The goal of this study was not to find the secret recipe for a highly successful TTO, since there was a great variety between the different TTOs studied. Instead, the researchers aimed to identify aspects that explain the high performance of individual TTOs. Then, they continued to study if aspects between different TTOs were overlapping, and if this could explain their success.

From the interviews, a TTO topology was developed describing two fictional TTOs (Figure 11.3). The topology describes two different types of offices: the common good and the entrepreneurial TTO. Each of the two types shows aspects that typically cluster at a TTO, but the offices studied showed characteristics of both types to a larger or smaller extent. The first type, the common good TTO, is a non-profit organisation that aims to make full use of scientific output to benefit society. It is typically part of a research institute, and it receives funding from this research institute or from other public grants. Its main tasks include the protection of scientists' intellectual property (IP), consulting and training (potential) academic entrepreneurs, and interacting with other local organisations to strengthen the regional ecosystem. Next to this, it performs international networking activities to extend its transfer capabilities.

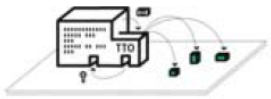
Type	Position and funding	Goals and practices
Common good 	Integrated Non-profit Subsidies from host organisation Third-party (state) funds	Benefits to host organisation and society (optimal) Knowledge transfer Dissemination opportunities IP protection Support to inventors upon request Mediation between inventors and (state) funding programs Opportunities for training and professional development (international) Collaboration with academia and industry Strengthening local ecosystem
Entrepreneurial 	Independent Own income State grants Earmarked foundation grants Start-up revenues	Economic impact (optimal) Commercialisation of findings and inventions Business opportunities Start-up investment Idea scouting Mediation between inventors and industry Staff with industry background Collaboration with industry Strengthening local economy and job market

Figure 11.3 TTO topology describing two fictional TTOs: the common good and entrepreneurial TTO. In reality, TTOs generally have characteristics of both types, but are more related to one of the two. Figure retrieved from Sinell et al. [176].

Contrary to the common good TTO, the entrepreneurial TTO is an independent organisation that aims to turn scientific findings into business opportunities. They do so by actively keeping an eye on scientific developments, and by scouting for ideas that can be turned into commercial success. Since the entrepreneurial TTO typically is a private organisation, they are able to invest their profits into new business opportunities. The responsibility over their own finances is also an incentive for entrepreneurial TTOs to actively strive for commercial breakthroughs. Entrepreneurial TTOs are often related to but not part of academic or other research organisations. Next to this, its employees often have an industrial background, since they have experience in how things are done on "the other side". It is mentioned that entrepreneurial TTOs mainly focus on their goal of gaining commercial success, by improving the local economy and by increasing the number of available jobs. The ways to get there, for example by setting up a start-up, are mentioned to be less relevant. In a way, entrepreneurial TTOs serve as a mediator between universities and companies. One challenge mentioned here is the differences in perspectives between the two, for example in terms of open science versus commercial protection. We will discuss this potential tension between open science and IP protection in more detail in Section 12.3.

Since both types of fictional TTOs are either integrated or associated with an academic or research organisation, it is placed towards academia on the academia-industry axis of the MCCS (Figure 11.1). On the innovation-collaboration axis, the model is placed in the middle, since TTOs facilitate collaborations between multiple partners in order to realise innovations.

At TU Delft, the I&I Centre is largely responsible for transfer activities. From the analysis we performed in Section 10.4, it follows that the I&I Centre best fits to the common good TTO, since it is a non-profit organisation that mainly assist researchers in their transfer activities and IP protection. Next to this, the mission of the I&I Centre, "Impact for a better society", fits to the description of a common good TTO. However, it also has some characteristics of an entrepreneurial TTO. This for example follows from the fact that both the current and former Business Cooperation Managers of Applied Sciences have a background in industry. Next to this, the I&I Centre has collaborations with companies. Other organisations affiliated to TU Delft that more closely resemble entrepreneurial TTOs are YES!Delft and Delft Enterprises (Section 10.4). YES!Delft is a business incubator that facilitates the formation of start-ups, characteristics that are typical to an entrepreneurial TTO. Although the main goal of Delft Enterprises is not to maximise profits (Section 10.4), it creates business opportunities and invests in the development of spin-offs. Together, the findings suggest that, at least in Delft, SynCellEU has the ability to perform activities that are either related to the common good or to the entrepreneurial TTO.

11.5.3 Science-technology-business ecosystem

In the first article describing an analytical model, Xu et al. distinguished three types of ecosystems: the science ecosystem, the technology ecosystem, and the business ecosystem [151]. Science and technology ecosystems were distinguished by the type of research performed and the research output: science ecosystems are concerned with fundamental research, which generates scientific knowledge, and technology ecosystems provide facilities for applied research, which is relevant for industry. Business ecosystems, on the other hand, consist of collaborations that are formed around a central technology. They are concerned with the applications that are relevant for this central technology.

Xu et al. described that the individual ecosystems have been studied extensively, but that the interactions between them and the innovation ecosystem that they form together are less elaborately explored. Therefore, the authors developed an analytical framework in which they linked the three different ecosystems into a science-technology-business ecosystem (S-T-B ecosystem, Figure 11.4). The inputs of the individual ecosystems are three quantitative indicators that are measured using value functions. These value functions are extracted from segments of the value chain and are specific to the innovation ecosystem under investigation. In the article by Xu et al., the 3D printing innovation ecosystem in China was studied. The value chain of this ecosystem was divided into the following four segments: "Design", "Materials", "Equipment Manufacturing", and "Services". These segments were sub-divided into 19 value functions, of which "3D Modelling Software", "Metallic Materials", "Ceramic Materials", and "Device Software" are some examples.

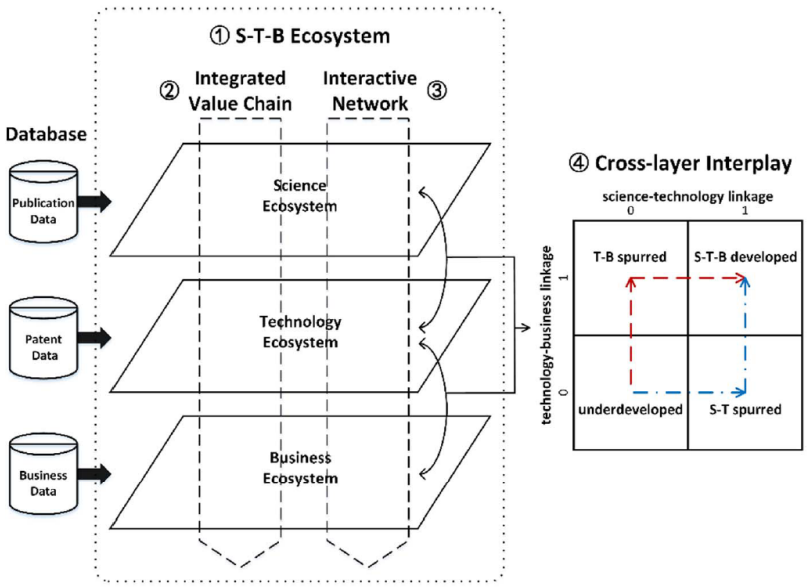


Figure 11.4 Analytical framework of the science-technology-business (S-T-B) ecosystem (1). Each ecosystem has its own quantitative input, and the layers are connected through the integrated value chain (2) and interactive network (3). The cross-layer interplay (4) depicts the potential development pathways of the S-T-B ecosystem, where ecosystems are either underdeveloped, T-B spurred, S-T spurred, or developed. Figure retrieved from Xu et al. [151].

The number of scientific publications on each of these value functions serves as the quantitative indicator of the science ecosystem (Figure 11.4). For the technology ecosystem, the number of patents that stem from each value function serve as input. Multiple indicators can be used as input for the business ecosystem, like sales and the number of companies working on a specific value function. The individual ecosystems can then be linked with each other through the integrated value chain and interactive network. In the case of the integrated value chain, a connection between the science and technology ecosystems exists when a value function is frequent in both layers. Similarly, links between the technology and business ecosystems are formed through the common prominence of value functions. In the interactive network, connections between the science and technology ecosystem are formed when a scientific finding is used to develop a novel technology, or vice versa. Links between the technology and business ecosystem are formed through commercial collaborations between partners from the two layers, that aim to bring a product to the market emanating from a novel technology.

Once the links between the individual ecosystems have been identified, the ecosystem type can be determined using the cross-layer interplay (Figure 11.4). According to this part of the model, there are two paths through which an underdeveloped ecosystem can become a developed S-T-B ecosystem. The first path, where ecosystems are technology-business (T-B) spurred before they become a fully developed innovation ecosystem, is mentioned to be a common route in developing countries. In these countries, innovations from developed countries are reproduced through reverse engineering. In this case, there is a low level of interaction with the local science ecosystem. In the science-technology (S-T) spurred ecosystem, on the other hand, there is a high level of interactions between the science and technology ecosystems, leading to the development of novel technologies. However, there are little interactions between the technology and business ecosystems in this case, leading to a low level of product commercialisation. Identifying the type of ecosystem can be useful to determine what strategies should be adopted to steer the ecosystem towards a fully developed innovation ecosystem.

The analytical framework developed by Xu et al. is placed in the middle of the MCCS (Figure 11.1), because it links universities with industry, and it studies both innovation (through the integrated value chain) and collaboration (through the interactive network).

In the model, no direct links between the science and business ecosystems are taken into account. These links were left out since in manufacturing industries, like 3D printing, the technology ecosystem provides a bridge between the science and business ecosystems, and direct connections between the latter two rarely occur. In the case of 3D printing in China, both science and technology ecosystems are centred at universities and research institutes, where fundamental as well as applied research is performed. The connections with the business ecosystem sprout form applied research performed in the technology ecosystem. 3D printing companies perform little research themselves, and are therefore the main actors of the business ecosystem. It is mentioned that direct science-business linkages are found more frequently in ecosystems based on the natural sciences, like bio-industries. Science-business connections could therefore be more relevant for SynCellEU. Due to the high level of fundamental and relatively low level of applied research performed by synthetic cell researchers, it is indeed likely that SynCellEU will form direct science-business linkages in the future. An example of such a link could be a university-industry collaboration where the company is responsible for both technology development and commercialisation.

The model is mentioned to be especially relevant for emerging technologies "since there is a high uncertainty about cutting-edge technology trajectories" [151] in this case, and there are more interactions between the science, technology, and business ecosystem compared to mature industries. This perfectly fits with the case of SynCellEU, where revolutionary applications are foreseen on the long term, but where the type of innovations and collaborations required to achieve those are still uncertain. Once SynCellEU has a clearer vision on what kind of applications they want to develop, they can potentially already make use of the "integrated value chain" part of the model. After identification of the value functions of a specific application, it could be determined to what extent this application is already under development by studying publication, patent and business data. The "interactive network" part of the model cannot yet be applied to the case of SynCellEU, since the level of collaborations with industry is still low. This part of the model, however, will become increasingly relevant as the ecosystem grows more mature. Once synthetic cell applications start to emerge, it will be interesting to apply the full model to identify stronger and weaker parts of the ecosystem, providing insights that can be used to steer the collaborations towards a fully developed innovation ecosystem.

11.5.4 The role of the local government on the innovation ecosystem

Through a case study on the Changzhou region in China, Ma et al. developed a model describing the influence of the local government on successful ecosystem development [150]. Changzhou is a city in the Eastern part of China, relatively close to Shanghai, and had 4.7 million inhabitants in 2018. The Changzhou region currently is a thriving local innovation ecosystem, with over one thousand high-tech companies applying and gaining authorisation for a large number of patents. In order to produce these innovations, companies are maintaining strong collaborations with universities and research institutes. Before the year 2000, however, only a few universities and research institutes were present in the Changzhou area. Through extensive investments and support of the local government to set up dozens of research organisations, as well as to facilitate collaborations between these research organisations and companies, the Changzhou region has grown to be a viable, dynamic, and rapidly growing innovation ecosystem in less than twenty years.

In the article by Ma et al., a descriptive model highlighting multiple stages in the formation of this innovation ecosystem is provided. In each of these stages, the local government had a different role in further developing the region. In the formation stage, the role of the government was to promote innovative talent with a top-down approach. They did this by setting up research organisations, facilitating inter-university collaboration and by organising extensive seminars, conferences and fora. At these activities, both research organisations and companies were involved.

In the second stage, the innovation ecosystem was further developed and improved. This was done partly by the establishment of financial organisations and service providers. They for example contributed to the enrichment of the innovation ecosystem by providing funding mechanisms and support for patent applications. Next to this, a platform for knowledge sharing was created. These activities mainly aimed to make the innovation ecosystem more independent, gradually shifting the role of the

government from a highly engaged to a more supporting actor. The third and final stage describes the expansion and internationalisation of the Changzhou innovation ecosystem. The local government enforced the supply chain integration of ten large sectors, among which the development of new medicines and carbon materials. International conferences were organised, which led to collaborations with multiple European countries. Next to this, thirty-one start-up incubators were established, contributing to the development of seventy key technology breakthroughs. This all happened roughly fifteen years after the activities to realise the innovation ecosystem started, indicating the importance to form university-industry collaborations at an early stage: research that is performed now, could lead to technology breakthroughs around the year 2035.

Since the model described by Ma et al. describes collaborations between universities and companies facilitated by the government, it is placed in the middle of this axis on the MCCS (Figure 11.1). On the innovation-collaboration axis, it is placed on the side of collaboration, since the policies discussed in the article are mainly about bringing parties together, rather than describing how they should actually innovate. The authors acknowledged that the extent of governmental influence on the innovation ecosystem might be different in other countries. For areas of future research, they suggest that innovation ecosystems in different cultural contexts should be studied, where not the government but another stakeholder is the driving force behind ecosystem development.

In the case of SynCellEU, universities are the central players that aim to form an innovation ecosystem. Contrary to the case of Changzhou, the number of universities and research institutes is not likely to be an issue. However, this does stress the question what elements might be lacking in an innovation ecosystems that is formed around universities, and what is required to overcome these hurdles. A similarity between the two cases is the usage of or desire for the coordination of an increasing number of activities. In the case of Changzhou, a Technology Management Office was set up to oversee the increasing number of knowledge sharing activities. Similarly, SynCellEU wishes that a coordinated instrument to oversee research activities becomes available in the EU (Section 9.3). The case of Changzhou is a successful example of how governmental coordination can indeed form a strong basis for an innovation ecosystem.

11.5.5 Living laboratory effects on innovation ecosystem development

Another model that is placed around the the middle of the MCCS is described in an article by Shvetsova and Lee [152] (Figure 11.1). The model studies the influence of living labs on innovation ecosystem performance. Living labs are places where innovations are developed with direct involvement of the end-user, often dealing with everyday problems. The model is placed around the middle of the MCCS, since universities and knowledge institutes are often involved in the innovation process as well, and it is increasingly recognised that living labs can be used to facilitate university-industry collaborations in innovation ecosystems [190, 191]. Examples of these university-industry collaborations are joint R&D projects, training programmes for company employees organised by universities, and the development of start-ups through university incubators.

The model is placed a bit towards industry on the academia-industry axis, because start-up partners were interviewed to develop the model. In living labs, innovation is achieved through collaboration, hence the placement of the model on the middle of this axis. The model is analytical in nature, where multiple living lab elements (e.g. source and organisational management) and their impact on innovation ecosystem development (e.g. human capital and infrastructure expansion) serve as inputs. By taking surveys from experts in the field, the importance of living lab elements and their influence on innovation ecosystem development can be extracted. The results of this survey can then be used to determine the influence of the living lab landscape.

The model was developed in the context of South Korea. This country is known for its innovative capacities, for example in terms of developing electronics (e.g. Samsung) and cars (e.g. Hyundai). Despite these technological developments, a gap between the scientific community and the industrial world has been identified by the South Korean government. The article by Shvetsova and Lee aimed to investigate if living labs could contribute to close this gap. To study the influence of living labs on

the development of the South Korean innovation ecosystem, the survey that serves as input for the model was taken by thirty start-up project managers from the living lab landscape. From this, it was found that living labs improve university-industry collaborations and have a positive effect on innovation ecosystem development. One limitation of this study was that all experts interviewed were active in the living lab landscape, potentially having a positive bias towards the effectiveness of living labs.

The model developed by Shvetsova and Lee studies the effectiveness of existing living labs in bringing universities and companies together. Since SynCellEU is not yet involved in any living lab activities, the model is not suitable to be applied to the case at this stage. It could, however, be interesting to investigate if living labs are a potential option for SynCellEU to start collaborations with industry.

11.5.6 PACES model

Another model developed by interviewing industry employees is the PACES model by Levrouw et al. [157]. The PACES model was formed in the context of an Industrial Design Engineering graduation project at TU Delft, and was later published in the cited scientific article. The project performed a case study on X!Delft, an organisation affiliated to TU Delft “aiming to ‘innovate together’ in a network of scientists, startups, students and corporations” [157]. Since its foundation in 2019, X!Delft aims to facilitate strategic partnerships between TU Delft and companies, thereby building an innovation ecosystem. They do so by facilitating partnerships between multiple industry partners at the university, without necessarily knowing what kind of innovations these partnerships will work on. The idea of these "open innovation initiatives" is that companies themselves can explore innovation opportunities for which they require collaborations with other parties, where they are not constricted to a specific innovation from the start.

At the start of the project, it was noticed that many industry partners were interested in this possibility of joint projects, but that little collaborative initiatives were taking place. This was attributed to two reasons. Firstly, it was difficult to find a project scope that fitted the interests of multiple partners. Secondly, companies were often reluctant to share organisational knowledge, which is required up to a certain degree in order to work together. With the goal to identify common drivers and restraints among industry partners to join a partnership, eleven semi-structured interviews were performed with company employees. From these interviews, five common challenges that were experienced among the different industry partners were found.

The first challenge that was identified is termed "Fear Of Missing Out" (FOMO), which is something that industry partners experience in a rapidly evolving world. It is about spotting underlying trends in science and technology that can be valuable to a company in the future. This FOMO can result in a certain level of stress when company employees feel like they are missing out on important and relevant information. Here, it is mentioned that universities can play an important role in soothing FOMO. By forming university-industry collaborations, companies can keep an eye on scientific and technological developments in academia. The second challenge identified is named "Future", which is mainly about being able to run a business today while keeping an eye on the future. In this way, this challenge is related to the first one, but it is more about the organisational factors that play a role in preparing for the future instead of a feeling to miss out. One example mentioned here is that Key Performance Indicators (KPIs), which are used to assess the performance of a company, often have a short-term focus and can therefore hamper long-term innovations.

The third challenge is "Flow", which is about the process of integrating a new technology into a company. This can either be a product or service, or an element of a novel innovation process. A concept that has close ties with this challenge is culture: companies often have a vision of how they want to internalise a certain technology, but a culture change is often required to genuinely achieve this. In this sense, the flow of a company should fit with their ambitions. The fourth challenge identified is "Family". This challenge relates to the previous one, where companies are often stuck in their flow and working culture. The idea of this challenge is that universities and industry partners can act as a family where they can learn from each other, not only on a personal level, but also in terms of how to tackle societal challenges. The fifth and last challenge identified is "Fun". It was noticed that company employees

often experience fun during innovation processes, “relating to human excitement for novelty and discovery” [157]. The challenge here is to find a balance between obtaining results while also having fun. If people are only focusing on doing things they like, the progression of the innovation process might be hampered.

Subsequently, the five identified challenges were combined into a single emotional driver, capturing both motivations and restraints for joining partnerships: suspense (Figure 11.5). The authors use the definition of Oxford University Press to explain this concept: *“a state or feeling of excited or anxious uncertainty about what may happen”* [192]. Suspense does not only capture uncertainty, which is related to the challenges of FOMO, Future and Flow, but it also includes excitement that people feel to work on new technologies in joint projects, relating to Family and Fun.

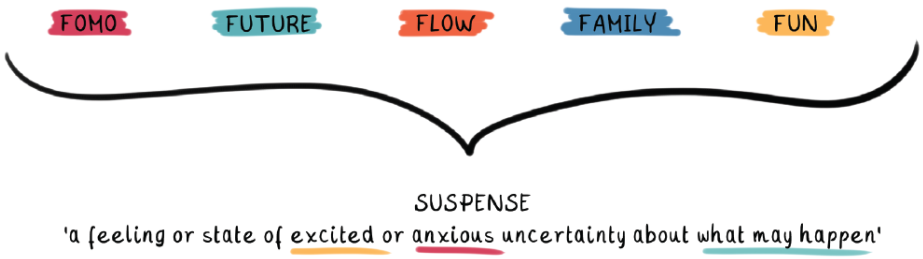


Figure 11.5 The five identified challenges in joining industrial partnerships at a university were combined into a single emotional driver: suspense. Figure retrieved from Levrouw et al. [157].

A challenge with suspense is that it has to be both created and preserved. People easily lose excitement when uncertainties take over, which could lead to inhibition of innovation progression. In order to make use of the feeling of suspense in innovation processes involving multiple partners, Levrouw et al. developed the Preserving And Cultivating Effective Suspense (PACES) model (Figure 11.6). This strategic model consists of six activities that a partnership should undertake in order to successfully translate suspense to successful innovations. The first three activities, perceive, perspective, and predict, are meant to sketch future scenarios that serve as a shared vision of the collaboration. By being open to new ideas and developments, collaboration partners perceive the possibilities of new innovation opportunities. These innovation opportunities can be taken into account in developing future scenarios, where the different perspectives of the parties involved should be taken into account. In this way, the scenarios will have a certain level of predictive quality.

The latter three activities, paraphrase, probe, and prove, are about turning the future scenarios into concrete actions through "backlogging", which is the process of “reasoning back from the future vision” [157]. For this, the scenarios should be paraphrased into action points, that for example consist of joint projects and probes. These probes are experiments where uncertain factors of the predictions are explored in a collaborative fashion. The idea of these experiments is to "learn by doing", and to get closer to realising the future vision through trial and error. Essentially, the aim of the probing activities is to find an innovation path that proves the predictions made.

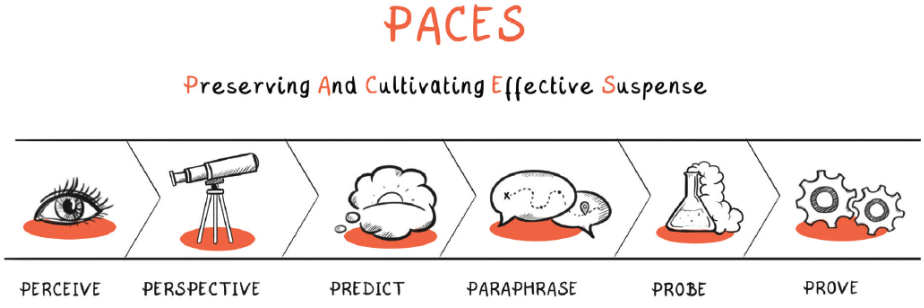


Figure 11.6 PACES model to preserve and cultivate effective suspense. Figure retrieved from Levrouw et al. [157].

The PACES model was developed with the goal to connect multiple industry partners at a university. Therefore, it is placed closer towards industry on the academia-industry axis of the MCCS, but not on the far end (Figure 11.1). Next to this, it mainly focuses on facilitating collaborations between different partners, but also takes innovation into account by sketching future scenarios. The model is therefore placed close to the middle of the innovation-collaboration axis, but a bit more towards collaboration.

Despite the fact that the model is developed from an industrial perspective, it could still be relevant for SynCellEU. The purpose of the model is to form industrial partnerships at a university, with the long-term goal to build an innovation ecosystem containing strong university-industry collaborations. By putting the initiative to form an innovation ecosystem at industry partners, it is, in a sense, formed the other way around than in the case of SynCellEU, where the initiative comes from academia. Here, the end goal of both collaboration formats is similar. To check if the case of SynCellEU fits into the PACES framework, it should be verified that the challenges on which the model was developed are experienced by SynCellEU as well, or that SynCellEU can assist companies in tackling the challenges they experience.

11.5.7 Triple-layer core-periphery framework of the enterprise innovation ecosystem

A descriptive model that is mainly developed from the perspective of a a company is the "triple-layer core-periphery framework of the enterprise innovation ecosystem". In this model, that is developed by Su et al. [153], two pre-existing models describing the role and position of an enterprise in an innovation ecosystem were combined. The first of these two models is the core-periphery framework that was adapted from Zhao and Zeng [193] (Figure 11.7a). In this model, a central node is identified around which the innovation ecosystem is formed. The party in the central node, in this case an innovative enterprise, facilitates collaborations between other partners involved in the innovation ecosystem, who are termed "the periphery". The other model is the triple-layer framework adapted from Chen and Gu [194] (Figure 11.7b). In this model, the innovation ecosystem is divided into three levels: the core layer, the platform layer, and the development-and-application layer. The core layer is similar to the central node of the core-periphery framework, and “guides all members in the ecosystem to innovate around a shared long-term aim and future” [153]. They do so by realising the platform layer, which serves as a link between the other two layers through joint R&D centres, financial and marketing services, and fora. The development-and-application layer consists of individual partners, like companies, customers, and universities, who make use of the platform layer to develop their own innovations. They are similar to the periphery of the core-periphery framework, and their innovation activities contribute to realising the shared vision of the innovation ecosystem.

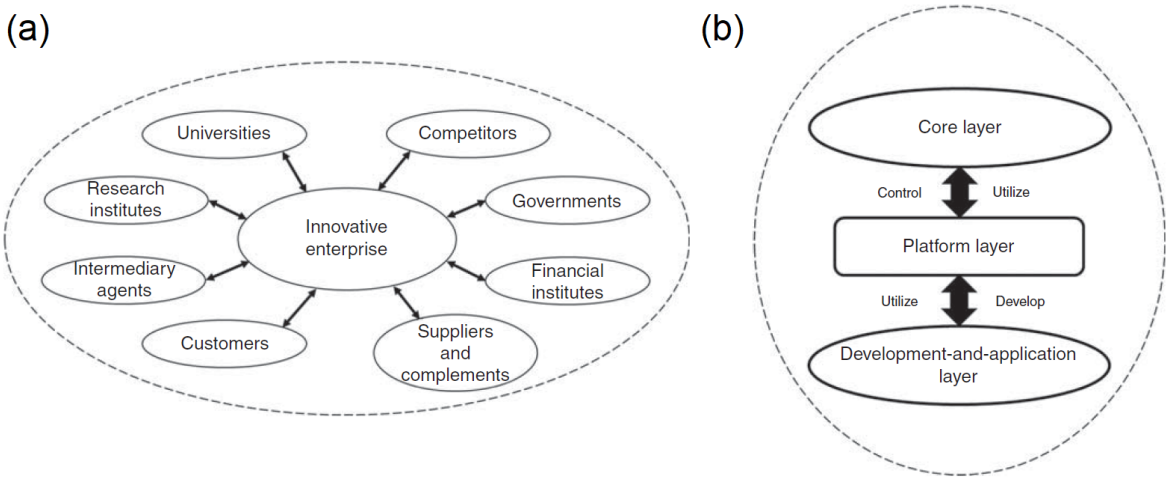


Figure 11.7 The core-periphery framework (a) and triple-layer framework (b) that are combined into a single model to describe the role of an enterprise in an innovation ecosystem (Figure 11.8). Figures retrieved from Su et al. [153].

Similarities between the two models are pointed out by the authors, but they were found to be “not illustrative enough without including each other’s information” [153]. Therefore, the authors combined the two models into a single triple-layer core-periphery framework (Figure 11.8). In this model, the core layer facilitates multiple platforms, where each platform interacts with its own partners in the periphery. The model acknowledges the complex architecture of innovation ecosystems, and allows for the exploration of multiple facets of this phenomenon. In the article, the framework is applied to Insigma Group, a major Chinese IT service provider. Insigma Group is actively engaged in innovative activities spanning over multiple technology platforms. They develop their own innovations in the innovation platform, where they actively collaborate with universities, research institutes and other enterprises.

Insigma Group also has an ideation platform that gives external individuals and teams the chance to realise their innovative ideas. The ideation platform organises creative competitions, where the most successful projects are given the opportunity to form a start-up at yet another platform: the entrepreneurship platform. This platform offers an incubator where start-ups can make use of the Insigma Group network to connect with universities, large companies and policymakers. A final platform that of Insigma Group is the financing and investment platform. Next to its financial responsibilities, it provides funding for remarkable start-ups from the entrepreneurship platform. In this way, the different platforms of Insigma Group’s innovation ecosystem are linked to each other, each interacting with their own relevant partners in the development-and-application layer.

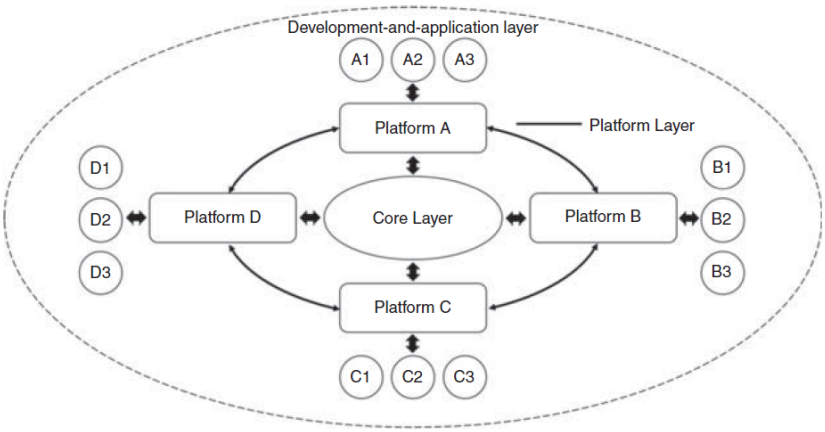


Figure 11.8 The triple-layer core-periphery framework of the enterprise innovation ecosystem. Figure retrieved from Su et al. [153].

Since the model is developed from the perspective of a company, it is placed towards industry on the academia-industry axis of the MCCS (Figure 11.1). Next to this, the model mainly describes the type of partnerships of an innovation ecosystem. Therefore, it is placed close to collaboration on the innovation-collaboration axis. The company under investigation has collaborations with universities, so the model is not placed entirely on the side of industry.

Although the model is developed to study the innovation ecosystem around an innovative enterprise, it could still be applied to SynCellEU once the innovation ecosystem grows. In this case, SynCellEU would be in the core layer of the model, and the platforms would consist of the different organisations that are required to realise the vision of SynCellEU. Examples of these platforms could be financial organisations and parties that promote entrepreneurial activities related to synthetic cell research. In the development-and-application layer, the relevant partners of each application would be present. Analysing the innovation ecosystem in such a way could become useful once the innovation ecosystem has become mature, to make an overview and explore links between increasingly complex collaborations. At this stage, however, the model is not yet relevant for SynCellEU.

11.5.8 Start-up survival model

In their work, Bandera and Thomas developed an analytical model through which they studied the influence of innovation ecosystems and social capital on start-up survival [167]. Here, social capital is a measure for the amount of relationships within a network. In their model, Bandera and Thomas distinguished between the availability of social capital (i.e. the amount of collaborations an organisation *could* form within an innovation ecosystem) and the utilisation of social capital (i.e. the amount of collaborations an organisation actually *formed* within an innovation ecosystem). Innovation ecosystems were considered as regional entities with the same ZIP code in this study. Within these innovation ecosystems, overlapping industries operate in collaboration with incubators, universities, and local governments. By considering innovation ecosystems on this regional scale, the effect of clustering social capital opportunities was taken into account in this research.

To develop the start-up survival model, the authors studied the survival of 4,928 businesses from the United States that were founded in 2004. First, the start-ups were classified based on their ZIP code and on the sector in which they were active. This resulted in an average start-up density per sector. Here, the density was higher when more start-ups working in the same sector had the same ZIP code, meaning that they were geographically located close to each other. Since social capital indicates the amount of relationships in a network, the start-up density was used to measure the availability of social capital in a specific region. One limitation of the study is that other stakeholders in the network, like universities and governmental organisations, were not taken into account while measuring the availability of social capital. To measure the actual utilisation of social capital by start-ups, these other stakeholders were taken into account. For this part of the analysis, the researchers studied if start-ups had collaborated with another company, a university, or a governmental organisation within a time frame of five years.

By investigating the influence of availability and usage of social capital on start-up survival, the researchers found that there is no correlation between the presence of social capital and start-up survival. This can be explained by the fact that the mere presence of social capital does not guarantee that start-ups make use of it. Indeed, the authors found no correlation between the availability of social capital and start-ups expanding their networks. On the other hand, making use of social capital strongly correlated with start-up survival. Here, a greater relationship was found for high-tech industries compared to low-tech industries. Although there are advantages for regions with a high start-up density, for example in terms of knowledge sharing through informal communication (Section 11.11), the results show that regional access to social capital is not essential to utilise it. In summary, the study shows that regional innovation clusters offer opportunities for business success, but that start-ups should actively engage with other organisations in order to increase their chances of survival, either within or outside their own region.

The analytical model developed by Bandera and Thomas studies the influence of social capital availability and utilisation on start-up survival. Since the model solely measures the amount of collaborations a start-up has, and does not take the innovation output of a company into account, it is put on the far right end of the innovation-collaboration axis on the MCCS (Figure 11.1). The model studies companies that collaborate with other companies, but also with universities. Therefore, it is put closer to the industry side on the academia-industry axis, but not on the extreme end.

Although SynCellEU is currently having little contact with start-ups, this might become more relevant in the future (Section 14.2). At that point, it will become relevant if it is beneficial for start-ups to locally cluster. We will elaborate on this question in Section 14.3 in the discussion.

11.6 Innovation ecosystem levels

The systematic literature review showed that there exists a great diversity in types of innovation ecosystems. Here, twelve articles described innovation ecosystems on a regional level (all articles except 4, 7, and 14), and nine articles recognised innovation ecosystems on a national level (all articles except 1, 2, 7, 9, 18, and 19). Note that some articles described innovation ecosystems both on a regional and a national level.

Su et al. rightfully mentioned that “not all innovation ecosystems have the same architecture and internal collaboration models” [153]. When describing different levels of innovation ecosystems, Su et al. referred to the article from which they adopted the core-periphery framework [193]. This article distinguished three types of innovation ecosystems, that either operate on the microscopic, middle, or macroscopic level. The microscopic level is concerned with innovation ecosystems orchestrated by organisations, like the one of Insigma Group described in Subsection 11.5.7. The middle level describes regional innovation ecosystems where specific types of industry are prominent. Examples of such middle level innovation ecosystems are the region of Changzhou (Subsection 11.5.4) and the description by Bandera and Thomas [167], where innovation ecosystems are classified based on their ZIP code (Subsection 11.5.8). Lastly, the macroscopic level is about national innovation ecosystems and the types of innovation and collaboration that exist there. Examples of innovation ecosystems operating on this level are the 3D printing innovation ecosystem in China (Subsection 11.5.3), and the living laboratory innovation ecosystem of South Korea (Subsection 11.5.5). Schiuma and Carlucci classify an innovation ecosystem as a regional entity, but they stated that it can be globally branched through connections between scientists and entrepreneurs working on similar real-world problems [149].

11.7 University-industry collaborations

One of the requirements of the systematic literature review was to find articles describing university-industry collaborations in innovation ecosystems. Consequently, all articles described university-industry collaborations at least to some degree. In the United States, an important event from the past that has resulted in part of today’s university-industry collaborations is the adoption of the Bayh-Dole Act. In their article, Silva and Ramos described how this law, that was accepted in 1980, allowed universities and research institutes to commercialise scientific findings that were made through governmental grants [159]. The enactment of the Bayh-Dole Act resulted in the foundation of the first TTOs, who started to act as a mediator in bringing universities and companies together. Through the development of TTOs, private investments in commercialising scientific findings became more frequent. This is especially relevant for the entrepreneurial TTO as described by Sinell et al. [176] (Subsection 11.5.2).

Silva and Ramos specifically discuss the influence of the Bayh-Dole Act on Academic Medical Centres (AMCs) [159]. They described that there are no known examples of drug approvals stemming from federal grants before Bayh-Dole, but that this frequently occurred after enactment of the law. In this way, innovation ecosystem are strengthened through university-industry collaborations. One argument they gave for this observation is that universities are possibly preferred over governmental organisations as an industry collaborator. Silva and Ramos discussed critique on the Bayh-Dole Act as well. Although part of the academics are fully in favour of commercialising scientific findings, others are worried that it might distract researchers from finding out the truth and performing the scientific method in the rightful way. This discussion is also interesting for SynCellEU. If commercialisation indeed distracts from pursuing scientific truth, SynCellEU’s investments in university-industry collaborations might hinder the development of fundamental knowledge. Although SynCellEU’s current focus still lies at performing fundamental research, this discussion might become more relevant in the future.

In their article on international experiences in university-industry collaborations [166], Kravchenko et al. referred to the results of a study carried out by the European Commission, called "The State of University-Business Cooperation in Europe" [166]. From this large-scale survey that was filled in by 17,410 respondents from 33 countries, it followed that the majority of universities and companies were

not involved in research-business cooperation. Although companies are increasingly recognising that collaborations with universities can be beneficial in terms of talent attraction and creating competitive advantage, ties between academia and industry were found to be weak and scattered. Both university and company employees agreed that shortages in funding and resources are the main factor limiting collaborations. Funding was indeed mentioned to be an important factor in developing the innovation ecosystem in other articles, for example by companies hiring PhD students through fellowship programmes (Schiuma and Carlucci, [149]), or by governmental funding for research centres (Ma et al., [150]). Sinell et al. described that the type of funding programmes that are available are a strong determinant for what kind of transfer activities are performed [176]. In this way, they influence the sort of collaborations that are formed as well.

Kravchenko et al. also identified differences between universities and companies in terms of challenges they experience in forming collaborations. It was found that researchers mainly see bureaucracy and time constraints as obstacles for collaboration, whereas company employees find differences in culture and incentives great hurdles. An interesting observation is that both university and company employees thought that the other party experiences more advantages from collaborations than they do themselves. This issue can possibly be solved through talent-exchange programs, that turned out to be a successful approach to foster university-industry collaborations in the case of Insigma Group [153] (Subsection 11.5.7). Related to this, Ma et al. pointed out that universities are "the key knowledge resource of the innovation ecosystem, as [they provide] training programmes for industry, and graduates with skills" [150].

The difference in timescales between academia and industry was identified as a challenge as well. Fischer et al., for example, indicated that companies often experience internal academic processes to be slow, thereby decelerating the formation of contracts [164]. A similar difference in timescales was pointed out by Dogterom between fundamental researchers and European policymakers (Section 10.1). On the other hand, Levrouw et al. described the difference in timescales between academia and industry as an opportunity, where universities can assist companies in keeping their eyes on the longer term [157] (Subsection 11.5.6). Similarly, companies can experience it as something positive when an academic employee provides them with a new perspective [195]. These strategies aiming to focus on opportunities instead of barriers are recommended by the European Commission in the conclusion of their report on the state of university-business cooperation in Europe [196]. At the same time, they believe that policies should focus on "providing conditions for establishing trust and setting common goals" [166].

In the interview with Dogterom, she indicated that they currently have little contact with smaller companies (Section 10.2). Lohle, however, indicated that start-ups can be required to set up the industry around new innovations (Section 10.2). In the studied articles, we found multiple examples that either showcased successful collaborations between smaller companies and universities, or that mentioned reasons why these type of collaborations are hampered. Kravchenko et al. explained that in the Siberian region of Novosibirsk, university-industry collaborations are especially important for starting businesses. However, none of the ten scientists interviewed by Kravchenko et al. mentioned "small business as an attractive partner in commercialization processes" [166].

Fischer et al. pointed out that the documentation and procedures required to form university-industry collaborations can be too labour-intensive for spin-offs [164]. As a result, partnerships with academia are less attractive for spin-offs once they have been formed. In their article on eco-entrepreneurship, that will be discussed in Subection 12.4.2, Saéz-Martínez et al. stated that the internalisation of additional assets, such as eco-innovation, increases with firm size [162]. It could thus be argued that the capability of companies to form collaborations with academia increase as they grow. Still, there are ways to foster partnerships between universities and smaller companies. Shvetsova and Lee pointed out that innovation transfer leads to "fewer entry barriers for new start-ups and small enterprises" to form university-industry collaboration [152]. Furthermore, Ma et al. stated that SMEs were able to interact with universities through governmental support.

11.8 Creating an entrepreneurial culture

Except for articles 11 and 23, all articles mentioned or discussed the importance of entrepreneurship in the development of the innovation ecosystem. In their research agenda, Schiuma and Carlucci suggest that more research is required on the role of universities in promoting entrepreneurial capital, which entails the capabilities and commitment of individuals or groups to perform entrepreneurial activities [149]. Here, they mentioned that universities play an important role in innovation ecosystems by providing a platform and by creating an atmosphere where entrepreneurial activities are promoted. The drive to innovate can possibly be encouraged by responding to people's excitement for novelty, which relates to the feeling of suspense as described by Levrouw et al. [157] (Subsection 11.5.6).

Multiple articles elaborated on the education and training activities that universities undertake to increase entrepreneurial capital (16, 17, and 28). Fischer et al., for example, discussed how the involvement of external organisations in the curriculum is beneficial to teach students the skills required to develop frugal innovations [164]. Frugal innovations are concerned with minimising production costs and complexity of applications, and will be further discussed in Subsection 12.4.3. Sinell et al. mentioned that students increasingly prefer social entrepreneurship over business entrepreneurship [176], where social entrepreneurship is more concerned with the common good TTO (Subsection 11.5.2), i.e. to develop innovations for society. It is important to take this preferences into account to tweak the type of entrepreneurial education available.

A more elaborate discussion on entrepreneurial education is provided by Gunn [165]. In her article, she specifically discussed education on bioentrepreneurship. Gunn described the myth that exists about biotech start-ups, where "a life scientist makes a breakthrough at the lab bench, meets a daring venture capitalist, and the two create a stunning biotech company" [165]. In fact, scientist require many differentiated skills to become a successful entrepreneur, for example in terms of pitching and applying for funding. Gunn highlighted multiple online and physical courses directed towards students from the life sciences to increase their entrepreneurial capital. One example is the Biotechnology Entrepreneurship Bootcamp, where the entire trajectory of developing a start-up is covered, from the first funding pitch to managing a bioenterprise. This example illustrates how education can contribute to the formation of start-ups.

Another example is the Master of Biotechnology Enterprise and Entrepreneurship, which is offered by Johns Hopkins University. The program can be compared with a Master of Business Administration focused on biotech, and is fully taught online. As a result, it "arguably serves multiple biotechnology ecosystems regionally, nationwide, and worldwide, and in many different ways" [165]. In the remainder of her article, Gunn presented a case study on the Bioentrepreneurship (BioE) program offered by the University of San Francisco. This university is part of a thriving biotechnology innovation ecosystem situated in the San Francisco Bay Area, where over eighty thousand employees work at more than one thousand biotech companies. The BioE program prepares students to become active in this innovation ecosystem through creative and exciting educational activities, such as biotech podcasts and global study tours. Since the BioE courses are available for all graduate students at the University of San Francisco, "the ability to serve the local biotechnology innovation ecosystem is possible on many levels" [165].

In the innovation ecosystem of Chinese firm Insigma Group, a whole entrepreneurship platform that promotes the formation of start-ups is present (Subsection 11.5.7). The importance of start-ups and spin-offs for the innovation ecosystem were also recognised in other articles. Xu et al., for example, wrote that spin-offs generally play a pivotal role in transferring scientific knowledge to applications in China [151]. From their analysis on the 3D printing network in China (Subsection 11.5.3), however, it followed that spin-offs "tend to be isolated in the network, with limited collaboration with other organisations" [151]. The identification of this missing link in the innovation ecosystem showcases the usefulness of their model; to improve ecosystem performance, 3D printing spin-offs should extend their networks.

In their article, Sinell et al. described mechanisms that promote the formation of spin-offs [176]. They

for example mentioned that some countries have national funding programmes to assist spin-offs [176]. Moreover, scientists often have a higher chance to obtain research grants if their experimental findings can result in technology transfer, which can be facilitated through spin-offs. Academic support in the formation of spin-offs was also found to be important.

The importance of business incubators for academic entrepreneurs was recognised by Fischer et al. [164]. These organisations that support start-up formation can assist academic entrepreneurs with managerial tasks and to extend their networks. Despite their added value to the innovation ecosystem, Shvetsova and Lee identified multiple reasons why the mere presence of business incubators and start-ups is not sufficient to enhance innovation ecosystem performance [152]. They stated that business incubators often form autonomous ecosystems. However, “the creation of an infrastructure, that is, a city, a region, or even an entire country, is required to effectively create an innovation ecosystem” [152]. Next to this, they brought up multiple reasons why start-ups often fail to be successful. Firstly, it is mentioned that start-ups are not capable of assessing societal needs and identifying target markets on their own. This indicates the importance of collaborations with an organisation concerned with these issues, such as the I&I Centre of TU Delft (Section 10.4). Secondly, they explained that it is challenging for start-ups to keep customers satisfied, since they have less customer data compared to larger companies. Thirdly, they mentioned that start-ups often experience difficulties in obtaining certifications required to enter the market, thereby losing innovative potential.

11.9 The relevance of competition for the innovation ecosystem

The relevance of competition for the innovation ecosystem was recognised by the majority of the articles (2-5, 7, 9, 14, 19, and 23). Liu et al. mentioned that, in the context of an innovation ecosystem, “companies rely on cooperation and competition to produce products, meet customer needs, and ultimately develop their innovation capabilities” [171]. Similarly, Ma et al. stated that competition is important for the development of new products and the innovation ecosystem itself [150], and Liu and Stephens attributed competitors to be key players of the innovation ecosystem [155]. Bandera and Thomas described that similar companies that are locally clustered in an innovation ecosystem both compete and cooperate [167].

In other articles, the role of competitive advantage was discussed. Xu et al. mentioned that by forming an innovation ecosystem, companies gain a competitive advantage over companies that are not part of the network [151]. Saéz-Martínez described that “universities can play a central role in designing competitive systems that are driven by environmental sustainability” [162]. The role of the university was also clarified by Levrouw et al., who stated that the an increased level of competition drives the formation of university-industry collaborations [157]. Shvetsova and Lee explained that, in South Korea, competition is a risk for start-ups who are active in fast-cycle markets, where innovations are not properly protected and are rapidly mimicked by larger companies [152]. Finally, Su et al. brought up the concept of "coopetition", where companies compete in becoming a larger player on the market, but cooperate on the formation, growth and protection of the innovation ecosystem [153].

11.10 The role of the government on innovation ecosystem performance

From many articles, it followed that the government played a role in the development of the innovation ecosystem. In this way, academia and industry are linked to the government as described by the triple helix model (Subsection 7.1.2). In the article describing the PACES model, for example, it was mentioned that through university-industry collaborations, “industry and universities are reacting to the government’s call to collaborate in favour of innovations that benefit society economically or socially” [157]. In a way, the Gravitation grant provided to BaSyC by the NWO is an example of responding to this government’s call. Although the funding from the NWO is mainly aimed to perform fundamental research, in the end it should lead to applications that have an impact on society. In the article by Liu and Stephens on implementing sustainability in the innovation ecosystem (discussed in Section 12.4), it is described that laws, for example related to environmental protection, are not always developed sufficiently for companies to invest in sustainability [155]. Here, the government could thus play an important role in developing these legal frameworks.

Next to these examples, it was specifically mentioned in the articles describing Asian case studies that the government had a great influence on innovation ecosystem performance. In the case about living laboratories and their effect on the South Korean innovation ecosystem, for example, it was the government who identified the gap between academia and industry (Subsection 11.5.5). Next to this, the South Korean start-up sector receives much support from both local and national governments.

The case of Changzhou is another example, where it was recognised by the authors that "the unique economic and social structure of China" have contributed to the formation of the ecosystem, where the local government had an essential role [150] (Subsection 11.5.4). Here, the government both promoted the foundation of dozens of knowledge institutes and facilitated university-industry collaborations through joint seminars and talent exchange programmes. In this way, the availability of social capital as well as the utilisation of it was realised. Especially the latter was found to be essential by Bandera and Thomas for start-up survival [167] (Subsection 11.5.8).

Bandera and Thomas described how attempts to duplicate successful examples of local innovation ecosystems, like Silicon Valley, often fail due to a lack of social proximity. Silicon Valley is a self-organised innovation cluster, where organisations voluntarily collaborate based on knowledge spillovers and similar social structures. Policies aiming to foster the innovation ecosystem by increasing the number of organisations should therefore also focus on promoting collaborations between these organisations. In this way, they can get used to each other's social structures, thereby facilitating collaborations. This is also an important point for Schiuma and Carlucci, who aim to bring prosperity to less developed regions by improving the innovation ecosystem [149] (Subsection 11.5.1). Here, social heterogeneity between different universities and companies should be taken into account while fostering collaborations between the two.

In the article where the Chinese firm Insigma Group is investigated (Subsection 11.5.7), it is also recognised that the government played an essential role in the development of the innovation ecosystem. They for example did this by setting up a large number of business incubators to promote entrepreneurship. Due to this high level of influence from the government, it was stressed that governments should be cautious and consistent in writing their policies. If policies are constantly changed, the development of the innovation ecosystem might be hampered due to companies having to adapt their strategies. Although the influence of the government is probably lower in Europe, a constant adjustment of policies could still impede the development of innovations, for example in the case of the European FET Flagships that were cancelled during the application process (Section 9.3). The efforts that SynCellEU put in this application are never fully wasted, but constantly having to change course due to new policies does come with a cost. This relates to the dynamical changes based on which SynCellEU constantly has to adapt its strategy (Section 9.2).

11.11 The importance of culture and informal communication for innovation ecosystem development

The importance of culture in the formation of innovation ecosystems was recognised by the majority of articles (only in articles 11, 17, and 19, the role of culture was not mentioned). Sinell et al. mentioned that knowledge and technology transfer is more successful if the involved parties are from the same country, thereby sharing cultural and social values [176]. Transferring knowledge and technology is more challenging when it involves multiple countries, since it "requires a deep understanding of how the foreign system works" [176]. This illustrates an additional challenge that SynCellEU faces in the development of a European innovation ecosystem. Sinell et al. not only mentioned transnational cultural differences, but also referred to a cultural gap between academia and industry. Here, it was mentioned that a tension exists between universities wanting to freely share scientific findings, whereas companies have commercial interests and often want to keep scientific findings confidential. Levrouw et al. described that culture plays an important role in the internalisation of technologies [157]. In the case of SynCellEU, this could be relevant in the integration of different synthetic cell modules. Finally, Ma et al. suggested the influence of national culture on innovation ecosystem development as an option for future research [150].

The importance of informal communication for innovation ecosystem development was recognised by six out of fifteen articles (1, 4, 7, 9, 18, and 19). Schiuma and Carlucci mentioned that innovation ecosystems comprise both formal and informal networks [149]. Empirical studies stressed the relevance of informal communication for high-tech companies, as pointed out by Bandera and Thomas [167]. Kravchencko showed that, according to the large-scale "The State of University-Business Cooperation in Europe" survey, informal interaction and knowledge sharing were considered as the most important factors to facilitate university-industry collaborations [166]. However, there was no general consensus in literature on the role of informal communication. Fischer et al. [195], for example, pointed out that it often takes multiple years before informal interactions are turned into genuine collaborations in the development of frugal innovations. Next to this, they mentioned that interactions between students and university employees of the investigated university are mainly informal, whereas they prefer a formal centralised communication channel.

A summary of the systematic literature review, including the most important points that will be further discussed in the following chapters, is provided in Figure 11.9.

Summary of Chapter 11: Systematic literature review on university-industry collboarations in innovation ecosystems

- The concept of innovation ecosystems is underexplored in literature.
- Innovation ecosystems are often related to the non-linear innovation model or helix framework. Practical examples of full implementations of these models remain scarce.
- Innovation ecosystems are part of dynamic environments, where sustainable interactions and strategy adaptations are required. The constant adaptation of strategies could impede innovation ecosystem development.
- A Model Classification Coordinate System (MCCS) is developed to classify models based on their focus on innovation, collaboration, academia, and industry. Models are also classified as either being descriptive, strategic, or analytical.
- In Section 11.5, the models identified with the systematic literature review are described. Each model is related to the case of SynCellEU at the end of each subsection.
- Innovation ecosystems are typically described on a regional or national level. Through connections between scientisits and entrepreneurs, they can be globally branched.
- University-industry collaborations in innovation ecosystems are mainly limited by:
  - A lack of funding and resources
  - Different cultures, perspectives, and timescales of academia and industry
- Factors limiting university-industry collaborations can be overcome by:
  - Focusing on opportunities instead of barriers
  - Promoting social proximity between university and company employees
  - Talent exchange programmes
  - Joint seminars, conferences, and symposia
  - Governmental support
- Entrepreneurship is important for innovation ecosystem developemnt. An entrepreneurial culture at universities can be promoted by:
  - Providing entrepreneurial education or training activities
  - The involvement of external organisations in the curriculum
  - Governmental and academic support for the formation of spin-offs
  - Business incubators supporting the formation of start-ups
- Collaboration and competition are both drivers for innovation ecosystem development.
- Similar cultural values and informal communication can drive innovation ecosystem development.

Figure 11.9 Summary of Chapter 11: Systematic literature review on university-industry collaborations in innovation ecosystems.

12

The implementation of RRI elements in the innovation ecosystem

Literature extensively described the need for RRI practices, but empirical evidence of successfully putting RRI into practice is lacking (Subsection 7.1.3). This also followed from an article identified through the systematic literature review, where Liu et al. mentioned that “most research [on responsible innovation] focuses on building conceptual frameworks and identifying key elements and dimensions, and there is lack of empirical studies” [171].

The difficulty of implementing RRI was also recognised by Dogterom. In her interview, she indicated that she experiences concepts like RRI or public engagement often as fields on themselves, and that she finds it challenging to go beyond the academic aspects of these concepts. She also mentioned that the theories on these concepts are not necessarily in synchronisation with the practical work floor: “*[RRI elements] are things you can talk about and you can agree on the fact that you think it is important, but when I’m just pipetting in the lab and my experiment is not working, that absorbs all energy, so to say. Then you’re not really thinking about ‘should I do this differently because of such considerations’, that is of course not the case. It is, however, important to think about it, to keep it in the back of your mind, also for people who are working on the bench. And to be able to respond at the moment when you are facing certain decisions where you should consider this. But it is notorious to be difficult, to do this properly next to each other. And the fact that it is important, it is very easy to agree on that, but then you still don’t have anything.*”

Dogterom also mentioned that she sometimes gets the idea that you can only make decisions once everyone has agreed on what you can and cannot do, but that you cannot work with that, because then you can never start anything. She thus indicated that it does not work to entirely fix what RRI means from the beginning, and that one should try to maintain the dialogue. Dogterom explained some of the themes of this dialogue: “*(...) how to organise that it is not just a few big players in the future, but that it is a technology that is way broader, also in the world. There are a few NGOs with whom we discuss this, that it is not only the rich Western countries that benefit from it first, but that the technology is used more inclusive. And more sustainable, that it is not again one that only uses. Nevertheless, that all sounds very beautiful, but how do you do that?*”

12.1 Selection of RRI elements

To address the need for more insights in the practical implementation of RRI elements, we will discuss several RRI themes in this chapter. The RRI elements we will discuss were extracted from the systematic literature review (Chapter 11), and are visible in Table 12.1. Here, it is displayed how many articles mentioned each RRI element. This was determined using the code-to-article matrix (Appendix G). It can be appreciated that each RRI element was named in seven to thirteen out of the fifteen selected articles, showing that the selected RRI elements are relevant to university-industry collaborations in innovation ecosystems. To verify that the selected RRI elements are indeed part of RRI, we related them to the six RRI dimensions described by Kwee et al. [137] (Subsection 7.1.3). We found that "public engagement" relates to the first four RRI dimensions of inclusion, anticipation, reflexivity, and responsiveness. This is because all these dimensions are about the early engagement of stakeholders, either focused on determining the values or activities of these stakeholders, or on the actions that

should be undertaken based on the insights obtained from early engagement. Next to this, "open science & open innovation" fits to the RRI dimension of openness and transparency, and "sustainability" itself is one of the six RRI dimensions.

To validate if the RRI elements are also relevant to the case, we determined the number of times a specific RRI element was discussed in the semi-structured interviews. Here, we distinguish between an RRI element that was brought up or recognised by the interviewee (Table 12.1). If an RRI element was mentioned by an interviewee without us initially asking about it, we counted it as "brought up by interviewee". If we introduced an RRI element in an interview question and its relevance to the case was confirmed by the interviewee, we counted it as "recognised by interviewee". In Table 12.1, it can be appreciated that each RRI element was discussed in at least half of the interviews.

RRI element	Mentioned in article	Brought up by interviewee	Recognised by interviewee
Public engagement	7/15	3/4	1/4
Open science & open innovation	13/15	1/4	1/4
Sustainability	9/15	2/4	0/4

Table 12.1 RRI elements were extracted from the systematic literature review, verified by relating them to the six RRI dimensions described by Kwee et al. [137], and validated with the interview transcripts. "Mentioned in article" indicates the number of articles that mentioned a specific RRI element. The remaining columns indicate the number of times an RRI element was brought up or recognised by an interviewee. Usai and Dogterom were interviewed together, resulting in the total number of four interviews.

Some models from the systematic literature review were specifically related to one of the identified RRI elements, or about the implementation of RRI in general. We will discuss these models in this chapter. Next to this, we will provide insights from other articles and from the semi-structured interviews. We will also relate the acquired insights to the implementation of RRI elements in a synthetic cell innovation ecosystem. Finally, we will provide a short discussion on the implementation of RRI elements in the formation of university-industry collaborations.

12.2 Public engagement

SynCellEU aims to collect the desires and concerns of the public related to synthetic cells at an early stage of development. For BaSyC, the Future Panel is studying how to implement RRI elements in the project (Chapter 1). During a semi-annual BaSyC meeting, the Future Panel presented their first findings. The main message of this presentation was that increased efforts for public engagement should be made, but how this should be implemented exactly was still unclear. They did indicate that they prefer a bottom-up approach, where people take the initiative themselves to organise public outreach activities. However, when one enthusiastic PhD student indicated that she wanted to work on public engagement and asked whom she should approach for this, no clear answer was given. This indicates that some top-down steering might be desirable, since this enthusiasm could otherwise perhaps be left unused.

12.2.1 Organising public engagement through living labs

A practical example of involving society in an early stage of the innovation process are living laboratories as discussed by Shvetsova and Lee [152]. Although living labs are less prominent in the natural sciences, they are more frequent in potential application fields, like medicine. The Dutch organisation Medical Delta, for example, oversees eleven living labs where universities, companies, health facilities and local governments work together on medical innovations [197]. Examples of these living labs are a research operation room, where the efficiency and safety of surgery is tested by doing real-time measurements, and the Phenomix Fieldlab, where people's health is assessed by studying metabolomic products. Once SynCellEU has a clearer view on what kind of applications they want to develop, living labs could potentially serve as interesting platforms to realise these innovations. Subsequently, the effectiveness of the living lab collaborations could be determined with the model developed by Shvetsova

and Lee (Subsection 11.5.5).

An example of public engagement that is more closely related to the case can be found at Bristol Centre for Synthetic Biology (BrisSynBio). In their article, Pansera et al. described how public engagement has been successfully internalised at this research institute [132]. The term RI was used instead of RRI in this article (see Subsection 7.1.3 for the difference in usage of these terms). We will therefore use the term RI as well when referring to the article of Pansera et al.

12.2.2 A case study on public engagement at BrisSynBio

BrisSynBio is part of the University of Bristol, a renowned British university that is “being ranked within the top 50 universities in the world in the 2018 QS World University Rankings” [132]. The university adopted a new strategy in 2016 with the goal to change its structure and increase its size. Although the terms RI/RRI are not specifically used in their strategy, it does contain many elements referring to RI/RRI, like notions on open science and gender equality. This relates to the statement that terms describing RRI elements change, but that part of the ideas described by these terms remain similar (Subsection 7.1.3). In 2016, the University of Bristol started efforts to build a new campus geographically close to the poorest areas of the city. The goal of this new "civic university" is to directly involve local communities in the research processes.

To study how public engagement is practised at BrisSynBio, semi-structured interviews were performed with twelve participants that are responsible for the RI programme of the centre. From these interviews, it followed that both the University of Bristol itself as well as the researchers involved in BrisSynBio already had experience with public engagement before the centre was founded. An example of this is the Public Engagement team of the university, that has been involved in multiple British and European projects focused on involving society in science. Due to the experience the founders of BrisSynBio already had, public engagement was practised at the centre from the moment it opened. During the development of the centre, they experienced a shift from the more traditional transfer approach of science communication towards methods focused on public dialogue. This transition corresponds to the general shift in science communication approaches that already started in the first decade of this decade [198]. The traditional approach, also termed "public understanding of science" (PUS), aims to inform the public about scientific findings, for example in the form of science cafes or science festivals. A critique on this approach is that by understanding science, one does not necessarily start to be engaged with it. According to the new belief, citizens only start to be genuinely engaged with science once they actively join the discussion on the impact of science.

BrisSynBio aims to achieve this form of public engagement in a two-fold manner. The first method is a top-down approach, which is aimed at “cultivating a culture of RI throughout the Centre” [132]. The goal of this approach is that all scientist at the research centre know the basis of RI, and understand how it relates to their own work and to the university as a whole. This is realised by organising monthly seminars, where external speakers give talks about a specific subject. At least four times a year, these seminars are related to RI. The motivation behind exposing natural scientists to RI flavoured talks is that research always has impact on society in one way or the other. In the article by Pansera et al., an interviewed philosopher exemplifies this by stating that it would not make sense if someone studying governance would not take society into account. Similarly, they believe that synthetic biologists can never be fully disengaged from the public [132].

Next to this top-down method, BrisSynBio uses micro-level approaches to practice public engagement. Part of these micro-level approaches are case studies that scientists have to perform, where their ability to estimate the societal impact of their own research is assessed. Another micro-level approach, that is involving the public in a more direct way, is through the Synenergene project. In this project, innovative and out-of-the-box ideas to perform public engagement are implemented, often in collaboration with artists. One example of a Synenergene project is a play that was developed by researchers, the Public Engagement team of the University of Bristol, and a local theatre. The play, that was called "Invincible", “puts the audience at the heart of some of the contemporary moral dilemmas presented by the development of Synthetic Biology” [132]. To develop the play, BrisSynBio scientists were asked to

philosophise about the kind of impacts their research could have on society in ten years. These ideas were processed into the play, where the audience was confronted with potential dilemmas related to synthetic biology that one might face in the future. After the play, scientists started a discussion with the general public about the moral dilemmas to which they were exposed.

Due to the successful performance of the play, multiple other activities related to public engagement were conducted. One example is a workshop led by artists where natural, social and humanity scientists reflected on the implementation of RI in the field of synthetic biology. It is reported that the participants started to perform more RI activities after the workshop, either in the form or organising events themselves or by exchanging RI related articles, “to reflect on how the ideas they explored together are surfacing in their day-to-day practice” [132]. Another example is the project PERFORM. In this project, starting PhD students were asked to critically reflect on their own research related to RI, and to explain this way of thinking to high school students.

Another important point identified in the study by Pansera et al. is that the implementation of RI should not be forced on people. BrisSynBio ensures that scientists have the ability to participate in RI activities by introducing them in creative ways, without demanding that people have to participate. This is in line with a statement from Dogterom in her interview. Here, she mentioned that it is a common way to think that every PhD student should work on public engagement, because it is important. This can for example be done by demanding that each PhD student writes a chapter on public engagement in their thesis. However, this will often lead to quite generic chapters that look like each other, since public engagement is not their main profession. People then put effort in it, but the field does not learn a lot about the implementation of public engagement. Dogterom also thinks that some people are naturally more interested in performing RRI related activities, and that it would be valuable to come up with something clever that at least part of the PhD students put more effort in it. Although Pansera et al. recognised that some scientists are more interested in RI than others, they also observed a difference among age groups. When comparing older scientists to starting PhD students at BrisSynBio, the latter generally show more interest in RI, which could be explained by the fact that they were exposed to it from the beginning of their career.

Still, it is noticed at BrisSynBio that not everyone is engaged with RI, and that it is generally the same people who are actively performing RI related activities. This is not necessarily a problem, but it could become an issue when RI gets isolated from other activities, for example focused on product commercialisation. If two separate groups are working on public engagement and commercialisation without effectively communicating to each other, it is unlikely that the wishes of society will be implemented properly in technology transfer activities.

Another challenge identified by Pansera et al. is to "persuade" scientists to invest time and resources in RI, since they already experience a high workload in supervising PhD students, publishing articles, and obtaining grants. A potential solution for this is offered by Sinell et al. [176], who suggest to let scientists adopt "hybrid role identities". It is important that universities offer the opportunity for people to participate in RI related activities, but this is not sufficient, especially for researchers whose time is scarce and who do not see the added value of it.

The interviewees from the article by Pansera et al. indicated that providing scientists with “time and clear and concrete examples of RI practices that have contributed tangibly to high-quality research” [132] are required to increase the efforts being put in RI. Unfortunately, finding good examples of RI implementations is something that researchers are struggling with. One of the reasons given for this is that the changes that scientists engaging in RI personally experience are cognitive and often intangible. The influence of RI initiatives is therefore often hard to quantify. However, examples are not fully lacking. One interviewee indicated that, since they have immersed themselves more in RI, they tried to get the "biology" out of "synthetic biology". They explained that, through dialogue with the public, they noticed that people are especially worried about scientists creating something alive that can replicate. They noticed that by working with *in vitro* systems where cells lost the ability to divide, people are generally less concerned. This might be especially relevant for SynCellEU, since they aim to build a synthetic cell that is able to replicate without external help. Maintaining dialogue is therefore essential

to discuss the development of a synthetic cell, taking the concerns of the public into account.

The case study on BrisSynBio by Pansera et al. provided us with some interesting lessons about public engagement and RI in general. However, it is acknowledged by the authors that the interviewees were generally in favour of RI related activities. Therefore, they could be biased towards the effectiveness of RI implementation. To obtain broader insights in the current state of RI internalisation in the field of synthetic biology, the authors suggest that more case studies in synthetic biology centres should be performed.

12.3 Open science & open innovation

In their interview, Usai and Dogterom recognised that RRI elements should play a role in forming collaborations with industry. However, related to sharing knowledge, they indicated that they also had discussions with the Future Panel about open science and no protection of patents. Open science is about the transparent sharing and free availability of knowledge [199, 200]. By making scientific publications broadly accessible, open science could indeed accelerate the pace in which innovations could be developed. However, it would not necessarily result in the most ethically sound innovations, as pointed out by Shelley-Egan et al. [141]. According to them, RRI and open science are two related concepts, both striving for "systematic change in the practice of research and innovation" and aiming to solve "grand societal challenges" [141]. However, they stated that the concepts are motivated by different agendas. Through their analysis of the two concepts, they concluded that RRI is mainly concerned with desirable outcomes, i.e. developing innovations that society requires. Open science, on the other hand, focuses on doable outcomes, meaning that it is more about optimising transfer processes. This distinction between the two concepts is an important point, especially due to the increasing European attention to open science, in a time where RRI seems to lose momentum. Although the two could go hand in hand, Shelley-Egan et al. pointed out that open science should build upon the foundations that RRI has created, rather than overtake it.

Chesbrough indicated that open science does not necessarily lead to open innovation [201]. Here, open innovation refers to the joint development of applications through co-production, where knowledge and insights are shared. Chesbrough made his point based on the observation that many initiatives for open science have emerged over the last two decades, but that this has not necessarily led to a comparable increase in innovation practices. He also pointed out the relatively low number of industrial applications that are developed in Europe compared to its strong scientific basis, which relates to the less-developed entrepreneurial culture in Europe as pointed out by Bovenberg (Section 10.4). This gap between open science and open innovation is partially explained by the great challenge in translating fundamental knowledge to concrete applications. Furthermore, IP plays different roles in open science and open innovation. In open science, the goal is to make scientific knowledge available to everyone. As pointed out by Chesbrough, "assigning IP rights during the scientific discovery process throws sand in the gears of open science, inhibiting the free exchange of ideas and knowledge that lead to faster, better science" [201]. However, the commercialisation of scientific findings through open often innovation requires, at least to some degree, IP protection. According to Chesbrough, the level of protection should be properly balanced. Here, some protection is required to stimulate investments in the innovation, but the background knowledge upon which the innovation is built should be freely available. This background knowledge can then subsequently be used by other scientists and entrepreneurs for research or to develop other innovations.

In the interview with Jacobs, she recognised that tension can arise between open science and IP protection, but that this is not necessarily the case. She explained that one can apply a finding for IP protection and make it public once it is accepted. Naturally, there is a time window in between the IP application and acceptance, but this typically does not take more than a few weeks to a few months, according to Jacobs. After this, utilisation of the patent can be negotiated.

12.4 Sustainability

12.4.1 Integrating sustainability in the innovation ecosystem

Prof. dr. Hub Zwart, who is a philosopher involved in BaSyC, proposed to steer synthetic cell research towards sustainable applications in a recent article on the website of SynCellEU [202]. The question how sustainability should be integrated in the field of the synthetic cell, however, remains to be explored. In the systematic literature review, we identified one article by Liu and Stephens specifically discussing the implementation of sustainability in the innovation ecosystem [155]. Liu and Stephens mentioned that the realisation of sustainability within companies and along supply chains has been studied extensively, but that literature is lacking insights in how to involve other stakeholders of the innovation ecosystem. The article therefore tried to find a link between sustainability and innovation, where all parties involved in the innovation process were taken into account. In their definition of sustainable innovation, the questions if society desires and accepts a specific innovation is taken into account as well, indicating the relatedness between sustainability and public engagement.

The authors indicated that, within a supply chain, it is essential that all companies involved invest in sustainability to genuinely make a product sustainable. A company can produce cell phones with a high-tech energy saving method, but if the raw materials required to make this cell phone are extracted in an unsustainable way, one does not end up with a fully green product. Still, making the entire supply chain sustainable takes time, and one should start somewhere. Sustainability is thus often not necessarily about producing novel sustainable products, but also about making the production process of existing applications cleaner. This relates to the point made by Usai in her interview, where she explained that companies are increasingly interested in technologies that can make current procedures more sustainable (Section 10.2). SynCellEU, which aims to solve grand challenges such as the energy transition, could thus contribute to this challenge by not necessarily developing new techniques to generate energy, but also by optimising current production processes. Since the availability of energy might become scarcer when we will fully convert to sustainable energy, ensuring that current production processes require less energy in the future will be a valuable contribution.

Next to the optimisation of existing supply chains, radical innovations require new supply chains to be formed. Although this offers the opportunity to make a supply chain fully sustainable, it also requires the formation of new collaborations within the innovation ecosystem. An important asset of an organisation leading the supply chain of a novel technology is therefore to facilitate relationships among the individual partners within the supply chain. Aspects that belong to this integration of the supply chain are to ensure that companies within the supply chain are both willing to share knowledge and are able to implement the obtained knowledge in their production processes. The party responsible for this supply chain integration is therefore important in realising new products, and is termed the "innovation inductor" by Carvalho and Barbieri [203]. For SynCellEU, it is an important question who will adopt this role as innovation inductor in the development of new applications. In the linear innovation model, scientific findings are transferred from the university to industry in a unilateral way (Subsection 7.1.2). Within this model, the university has little to no involvement in the supply chain. Since SynCellEU aims to perform knowledge co-production in the future, however, their role in the supply chain might become more relevant. They should therefore start to think about the question if they see themselves fit as an innovation inductor, or if they prefer to outsource this role to a future collaborator, for example a large company that is more directly involved in the supply chain.

To develop a conceptual framework on sustainability in innovation ecosystems, Liu and Stephens performed three exploratory case studies. These case studies were executed by studying the sustainability initiatives of three companies: Heineken, BMW, and P&G. For this, secondary sources such as company websites, events and application development processes were studied. A similarity between the case studies is the identification of an innovation platform for each company under investigation. Heineken, for example, has set up a "Innovators Brewhouse", where the challenges that Heineken faces are discussed with scientists, entrepreneurs, collaborating companies, and the public. In the field of sustainability, the initiative is for example invested in exploring sustainable materials for packaging. In the case of BMW, public engagement is performed through their "Co-Creation Lab", where

citizens can interact about their ideas for the car of the future. Activities organised by the Co-Creation Lab include innovation contests, where the public is for example asked to design their own car interior. In this way, BMW is taking the wishes and desires of the public into account. The consumer products company P&G has set up a "Connect+Development" model, where external stakeholders and people can discuss and share novel technologies and resources. Next to this, the development of sustainable technologies by the companies are discussed, which include electric cars and powerful batteries in the case of BMW, and recyclable materials in the case of P&G.

The exploratory case studies were combined in a conceptual framework describing the relevant stakeholders and requirements to integrate sustainability with innovation. This framework consists of a loop where sustainability in an ecosystem is a driving force for innovation by bringing parties together, and where this innovation leads to the development of sustainable applications. Although Liu and Stephens are novel in their approach to link sustainability with the innovation ecosystem, the exploratory case studies only touch the surface of this topic by briefly describing the collaborative sustainability activities of the three companies. To genuinely grasp how partners can promote sustainability in a joint effort, in-depth case studies are required to identify the challenges and uncertainties that companies face in this process. This need for more elaborate case studies is addressed by the authors themselves as well [155].

12.4.2 Drivers of eco-innovation

In their article on eco-entrepreneurship, Sáez-Martínez et al. presented their descriptive model on eco-entrepreneurship (Figure 12.3), which covers drivers for performing entrepreneurial activities focused on sustainability [162]. They proposed these drivers for eco-innovation by studying the general concept of innovation, specifically the technology-push and market-pull innovation models (Subsection 7.1.2). Here, the technology-push model is represented by the supply side in the model, and the demand side stands for the market-pull model. The drivers of the supply side are about an expected scarcity in energy and materials in the future, which is likely to result in higher prices. Similar to the market-pull model, the demand side is about the desires of the public, that increasingly demand green solutions.

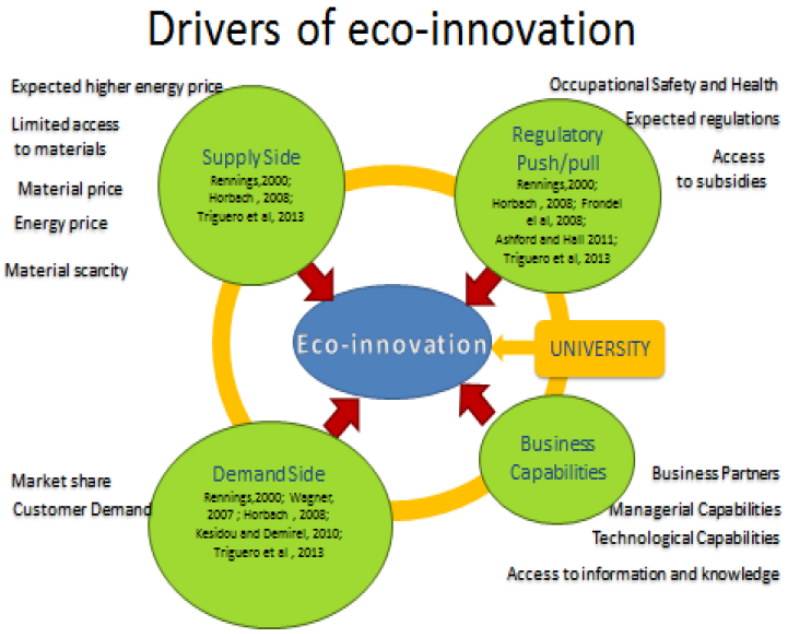


Figure 12.1 Drivers for performing entrepreneurial acitivities focused on sustainability. Figure retrieved from Sáez-Martínez et al. [162].

Next to the demand and supply sides, two other frameworks were incorporated in the model. The first one is the regulatory push/pull model, which entails current and expected future policies. In the context of sustainability, these entail environmental policies and regulations. These were found to be relevant for the model, since they can force companies to invest more in sustainable applications. Moreover, companies themselves are often incapable of estimating the economical advantages of investing in sustainability [204]. Finally, there is the driver related to business capabilities, which is about the availability and utilisation of human capital and knowledge resources by companies. Together, these four drivers influence how entrepreneurial universities are able to contribute to sustainable innovations.

The model on eco-entrepreneurhip was tested by using the results from the the Flash Eurobarometer survey, which contains data from over five thousand interviews with randomly sampled SME managers from twenty-seven EU countries [205]. Using this data, Sáez-Martinez et al. studied how their identified drivers for eco-innovation played a role in the development of sustainable products, services, processes, and (organisational) methods. Through their analysis, they found evidence for three of the four drivers contributing to the development of eco-innovations - only for the regulatory push/pull driver, no significant effect was found. Another observation the authors made is that the incentives to perform eco-entrepreneurship increase with company size, which was discussed in Section 13.2. Moreover, it was found that university-industry collaborations positively influence the realisation of sustainable innovations. This finding is attributed to EU funding for fundamental research. In this way, SMEs are able to develop innovations based on fundamental research. As the authors put it, “it is through cooperation with universities that SMEs can access leading edge research as universities are considered SME’s R&D departments” [162].

The authors conclude with some recommendations in terms of strategic policies and future research. In terms of policies, they suggest that national policies should follow the European example in promoting university-industry collaborations, since “national policies based on subsidies and fiscal incentives seem to be ineffective” [162]. This could be relevant for SynCellEU if they want to from future collaborations with companies to develop sustainable applications. For future research, the authors propose to further study the link between the university and the identified drivers for eco-innovation. SynCellEU could use insights from this future research to determine how they should formulate their sustainability strategies.

12.4.3 Frugal innovations

In their article, Fischer et al. discussed the elements required to develop frugal innovations [164]. As described in Section 11.8, frugal innovations focus on reducing costs and complexity in the development process. Fischer et al. conducted a case study on the University of Campinas (Unicamp), “one of the leading universities in Brazil in terms of research quality and technology transfer” [164]. Frugal innovations are especially relevant for developing countries, since they allow applications to become available to a broader public. However, they are also relevant for more developed countries, since they could contribute to the development of sustainable innovations. Reducing production costs for frugal innovations is often motivated by resource scarcity, which was mentioned to be a driver for eco-entrepreneurship by Sáez-Martínez et al. (Section 12.4). To learn how Unicamp has adopted strategies to promote the formation of frugal innovations, fourteen university experts were interviewed, which included technology transfer professionals. One of the hurdles identified through these interviews was the lack of rewards and incentives for developing frugal innovations. It was mentioned that the current incentives to develop frugal innovations are related to classical third mission activities. These incentives for example include IP protection, the promotion of spin-off formation, and forms of advice to increase the revenue of those spin-offs. Fischer et al. suggested that incentive structures should be redesigned in order to better fit with the view of frugal innovations [164]. The authors do not comment on how these incentive structures should be redesigned.

By combining the insights Fischer et al. obtained through the interviews, they developed a conceptual model describing interactions between stakeholders and activities required to realise frugal innovations (Figure 12.2). Here, the parties and activities that were found to play an important role in the development of frugal innovations are displayed, as well as the relationships among them. At Unicamp, for

example, TTOs were set up to “reduce the mistrust between the university and firms” [164]. Currently, technology transfer plays an important role in identifying research directions that could contribute to frugal innovations, and by linking these scientists to the right companies. This is similar to the role of the I&I Centre of TU Delft described by Lohle (Section 10.4), where they match research with the wishes of the outside world. In Section 11.8, we described that the involvement of external organisations at Unicamp resulted in students learning the skills required to develop frugal innovations. Moreover, a large number of spin-offs sprouted from a sustainability research project at Unicamp, which involved strong university-industry collaborations. The model shows both linear innovation processes (indicated by unidirectional arrows in Figure 12.2), as well as non-linear innovation processes (indicated by a bidirectional arrows in Figure 12.2), which fits to the description of Caryannis and Campbell that both type of innovation processes occur in parallel in an innovation ecosystem (Section 7.1.2).

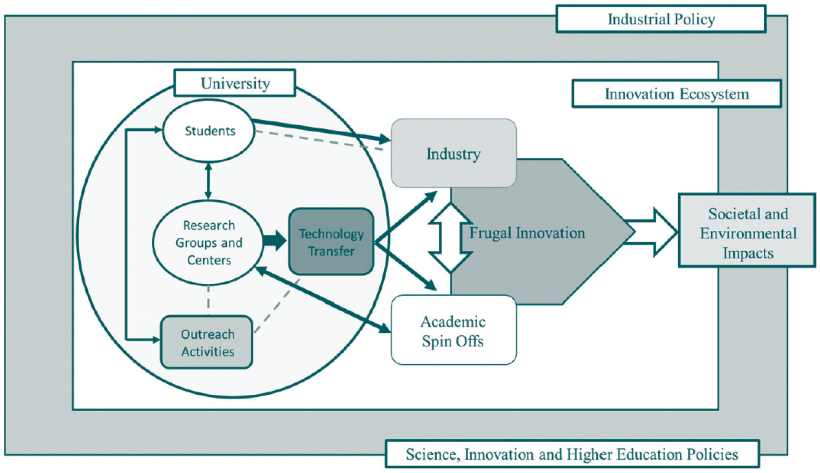


Figure 12.2 The conceptual model on the implementation of frugal innovations as proposed by Fischer et al [164]. Unidirectional arrows indicate one-way interactions, and bidirectional arrows indicate two-way interactions. Dashed lines indicate weak bonds. Students are, for example, linked to industry by joining a company after their graduation (strong bond) and through connections between student organisations and companies (weak bond). Figure retrieved from Fischer et al. [164].

12.5 Implementing RRI elements in the formation of university-industry collaborations

In innovation ecosystems, several stakeholders like universities, companies, and governmental organisation collaborate in order to translate scientific findings into commercialised products. Levrouw et al. indicated that this requires university-industry collaborations to be redesigned, where mere knowledge transfer and exchange is extended to the joint creation of knowledge [157]. In this process of knowledge co-production, applications are developed in a collaborative fashion from the start. In a way, building an innovation ecosystem itself can thus be seen as an RRI approach, since it requires stakeholders to form collaborations in an early stage. This fits to the RRI dimensions of inclusion, anticipation, reflexivity, and responsiveness as described by Stilgoe et al. [136] (Subsection 7.1.3). The aim of SynCellEU to build an innovation ecosystem therefore fits with the increasing demand for attention to RRI related themes.

In his interview, Bovenberg gave some clarifications on whether RRI elements are already taken into account when a university starts a collaboration with a company. He thinks that people do not always talk about these elements concretely, but that many aspects of it come back in a business plan. In this business plan, companies for example try to identify whether there is a demand for a certain product. Another consideration that has to be made for this business plan is what kind of technologies will be used. Here, rules and laws and the broader acceptance of the specific technologies are also taken into account. Bovenberg indicated that for synthetic cell research specifically, questions like “*what is responsible?*”, “*what is safe an what is unsafe?*”, and “*what is valuable to whom?*” are important to be addressed. He also pointed out that parliamentarians in Brussels have agreed on RRI implementation in the New Horizon programme for research, development and innovation. As a consequence, they have the responsibility that this really occurs. They can do this by ensuring that funding ends up at the

right place, for example by providing grants to projects that have concrete plans on how to implement certain RRI elements. In this way, the effects that are aimed for are genuinely realised. Bovenberg acknowledges that this is perhaps an ideal world scenario, but he does believe that European projects, in the long run, cannot get away with saying that they are going to implement RRI without having a concrete plan on how to do this.

12.6 Implementing RI in the shared bicycle innovation ecosystem

Through the systematic literature review, we found one article by Liu et al. presenting a model on the implementation of RI in the innovation ecosystem [171]. This model does not describe one specific RRI element, but is about the implementation of RI in general. In this article, the term RI is used instead of RRI. We will therefore use the term RI in this section as well.

The article by Liu et al. specifically discussed the implementation of RI in the shared bicycle industry in China. Initiated by four graduate students in 2014, the shared bicycle industry rapidly grew with almost 19 million users by late 2016. Liu et al. specifically studied one shared bicycle brand, Hellobike, and how it implemented RI in the industry in four stages (Figure 12.3). In the first stage, "Formation", the industry had one-way relationships with the government and users. This stage mainly consisted of brand development, small scale tests, and an exploration of societal needs. Since the shared bicycle industry in China was initiated from bottom-up activities, legal frameworks were lacking. The government therefore had to take a leading role during the second stage, "Expansion", where standardisation of the industry was developed as it increased in size. In the third stage, "Convergence", bilateral collaborations between the government, industry, and users were formed. Here, the latter two for example interacted at promotional events, or through a new credit system “improving user awareness of responsibility in a tangible way” [171]. In the fourth and final stage, "Renewal", bilateral university-industry collaborations were formed to safeguard future technological developments of shared bicycles.

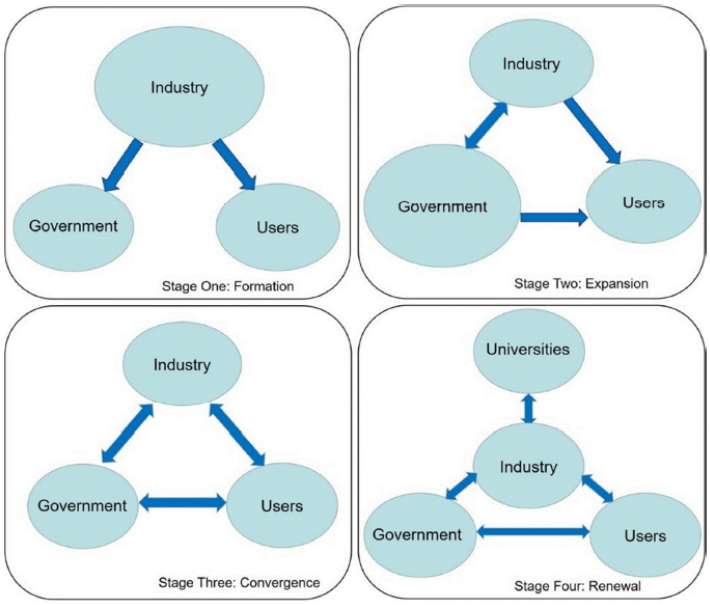


Figure 12.3 Descriptive model on the implementation of RI in the shared bicycle innovation ecosystem in China. Figure retrieved from Liu et al. [171].

The model shows that the government only started to interact with the industry once the industry started to grow. The government for example had to respond to nuisance of randomly parked bicycles blocking pavements. Although the model has a very specific scope, it does illustrate the importance to form collaborations, in this case between industry and the government, before problems start to arise. Similarly, for SynCellEU, it is important that potential implications of synthetic cells are addressed at an early stage of development.

A summary of the descriptive literature review on the implementation of the identified RRI elements is provided in Figure 12.4.

Summary of Chapter 12: The implementation of RRI elements in the innovation ecosystem

- From the systematic literature review, we identified three RRI elements that are relevant for university-indystry collaborations in innovation ecosystems: **public engagement**, **open science & open innovation**, and **sustainability**.
- Each identified RRI element corresponds to one or multiple of the RRI dimensions described by Kwee et al. [134]. Through the semi-structured interviews, we verified that the identified RRI elements are relevant to the case.
- Public engagement can be organised through living labs, but this only becomes interesting for SynCellEU once synthetic cell applications start to emerge.
- Public engagement is successfully internalised at BrisSynBio by:
  - Earlier experience with public engagement
  - Making RI-related activities available to everyone
  - Performing case studies
  - Organising events such as plays and workshops
- A common reason for scientists to not participate in public engagement is a lack of time, which could possibly be solved by allowing hybrid role identities.
- Clear and concrete examples of the effectiveness of RI-related activities are lacking. This could be explained by the cognitive and intangible changes scientists engaging with RI experience.
- RRI focuses on desirable outcomes, whereas open science focuses on doable outcomes.
- Open science does not necessarily lead to open innovation and requires a properly balanced level of IP protection.
- Large companies develop sustainable products through engagement with external stakeholders. The role of the universitiy in developing sustainable products requires more in-depth case studies.
- TTOs can serve as a mediator between universities and companies in the development of frugal innovations.
- SynCellEU's vision to build an innovation ecosystems requires the early formation of collaborations, and can therefore be considered as an RRI approach in itself.
- RRI elements are often discussed in a company's business plan, and can thereby be taken into account in the formation of university-industry collaborations.

Figure 12.4 Summary of Chapter 12: The implementation of RRI elements in the innovation ecosystem.

13

Application of a model to the case

13.1 Model selection

One of the goals of the systematic literature review (Chapter 11) was to identify a model that can be applied to the case of SynCellEU. In this way, SynCellEU can learn from previous experiences in their vision to build an innovation ecosystem, and adapt their strategies based on these lessons. Ideally, we would select a model that is developed from an academic perspective, since SynCellEU is managed by scientists and university directors. When consulting the MCCS, we only see three models or model elements that have an academic focus (Figure 11.1). Two of these are model elements part of the research agenda proposed by Schiuma and Carlucci [149] (Subsection 11.5.1). The model elements of Schiuma and Carlucci focus on activities and organisational units required to make universities more entrepreneurial. It is postulated that this would lead to an increased number of university-industry collaborations. Schiuma and Carlucci state that more knowledge on these processes is required, and they propose a research agenda [149]. The questions that are asked in this research agenda are highly relevant for SynCellEU, since they try to form university-industry collaborations from an academic perspective as well. However, since the answers to the questions are not provided by the model of Schiuma and Carlucci, we did not find it useful to apply it to the case of SynCellEU at this stage.

The other model closer to academics than industry on the MCCS is the TTO topology described by Sinell et al. [176] (Subsection 11.5.2). This model could be valuable if certain essential transfer resources were lacking for SynCellEU. We could then, for example, make suggestions on what type of institutes or activities should be set up in order to increase the transfer capabilities of SynCellEU. From our analysis, however, we found that characteristics from the common good TTO as well as the entrepreneurial TTO were both present at different institutes at TU Delft (Subsection 11.5.2). We therefore think that it is not useful to further apply the TTO topology to the case of SynCellEU. Possibly, the transfer possibilities at other universities or research institutes linked to SynCellEU are limited, but the identification of these possibilities lies beyond the scope of this study.

With no other academic model available, we started to postulate what other model characteristics would be relevant in applying it to the case. Here, we came to the conclusion that it would be interesting to apply a strategic model. In this way, SynCellEU can adapt their strategy to form an innovation ecosystem based on the outcomes of the model. Lessons learned from the descriptive models that are relevant to the case of SynCellEU are already discussed in Chapters 11 and 12. Since the descriptive models only illustrate the formation of innovation ecosystems, we can not further apply them to the case. Analytical models, on the other hand, will become more interesting once the innovation ecosystem has been formed; the performance of the innovation ecosystem, and potential missing links, could then be assessed with one of the analytical models.

The only strategic model or model elements left are two perspectives of Schiuma and Carlucci's research agenda, and the PACES model developed by Levrouw et al. [157] (Subsection 11.5.6). In the beginning of this section, we already discussed why we believe that the research agenda cannot be applied to the case. Although the PACES model is mainly developed from an industrial perspective, it could still be interesting for the case of SynCellEU. In Subsection 11.5.6, we already described that the innovation ecosystems envisioned by Levrouw et al. and SynCellEU have the same end goal, but a different starting point. Despite this difference, we believe that Levrouw et al. suggested an interesting

approach to foster university-industry collaborations, complying to the early formation of university-industry collaborations. Moreover, all developers of the PACES model are employed by TU Delft, the university where this thesis is written. This offered opportunities to easily validate if the PACES model is indeed applicable to the case with the developers themselves.

13.2 Validation of the model applicability

Before we contacted the developers of the PACES model, we validated if the identified challenges in forming partnerships are also relevant for SynCellEU. For the first two challenges, FOMO and Future, the academic basis of SynCellEU can serve as an interesting asset for potentially interested companies. SynCellEU is working on long-term technologies, and can therefore provide insights in products of the future to the industrial world. In this way, companies are able to prepare for the future, while running today's business in parallel.

In terms of Flow, one great challenge for SynCellEU is to integrate different parts of a synthetic cell into a complete working system. An example of this is the Dutch research collaboration BaSyC, that aims to integrate different cell modules with each other in one of their work packages (Section 1.1). Although these research groups are highly specialised in studying specific fundamental parts of the cell, integrating them with each other might be something in which they are less skilled. Next to the technical difficulties of this work package, it requires clear agreements and concessions on what individual modules will be used from which research group, and what findings will not be used for the final synthetic cell. Especially large companies will probably have more experience in combining insights from different groups to create a single integrated product. This relates to the fourth challenge of Family, where academia could learn from companies in terms of project management to build an integrated synthetic cell. Similarly, with their high level of fundamental knowledge, scientists could assist companies that perform research with issues they encounter.

The fifth challenge, experiencing human excitement for novelty while safeguarding results, could also be relevant for SynCellEU. Fundamental researchers are a textbook example of people that are typically driven by discovering the unknown, and by contributing to the knowledge base we as humans have. Although gaining fundamental knowledge definitely is a result, it only becomes genuinely interesting for society once useful applications are developed from it. Making this translation from fundamental knowledge to society might be something that fundamental researchers experience to be less enjoyable, but it is something that should still occur for innovations to be realised. The feeling of suspense can be linked to SynCellEU as well. Building a synthetic cell innovation ecosystem is a highly uncertain process, where the strategy constantly has to be adapted to the dynamic circumstances (as pointed out in the interview with Usai, Section 9.2). At the same time, it is an exciting new field where many promising applications are foreseen. Bovenberg specifically mentioned the importance of cultivating interest and creating an exciting climate that people want to be part of (Section 9.4), which can be linked to the process of cultivating effective suspense.

Lastly, Levrouw et al. stated that "it needs to be researched how the innovation potential created in the ecosystem can be translated into implemented innovations - as this seems to be a persistent challenge in innovation projects" [157]. This greatly resembles the challenge SynCellEU faces, where revolutionary applications are foreseen on the long term, but many uncertainties exist about how to get there. Together with the relatedness of the five identified challenges with the case, the PACES model shows promising applicability to the case of SynCellEU. By applying the model to the case, it can potentially be identified what future applications are interesting innovation paths for SynCellEU, and what kind of collaborations they require to realise these innovations.

To further validate the applicability of the PACES model to the case of SynCellEU, we had conversations with two of the developers of the model: Leonie Levrouw and Zwanet van Lubek. The PACES model was developed as part of the graduation project of Leonie Levrouw, and Zwanet van Lubek, head of Corporate Innovation at TU Delft, was one of her supervisors. During the development of the PACES model, they had the goal to let the involved companies come up with an innovation to work on. Here, the idea was that the companies would work on an innovation through knowledge co-production,

where all IP would be shared. Levrouw and Van Lubek, however, experienced that it takes a long time before companies and a university are used to working together. For this reason, the PACES model has not been tested yet. Currently, the Corporate Innovation department of TU Delft has set up a traineeship called X!Delft. Participants of this traineeship perform internships at both start-ups and larger companies, thereby exposing trainees to a great variety of businesses. With this traineeship, Corporate Innovation aims to form a university-industry network, possibly smoothing future collaborations.

To still be able to form a collaboration at this stage, Levrouw and Van Lubek identified common needs among the involved industry partners. Through this process, they found that companies are reluctant to collaborate if the subject of collaboration involves their core business. In that case, they are not willing to share their findings, since this might jeopardise their competitive advantage. Levrouw and Van Lubek therefore had to find a theme that was common among all companies involved, but not their core business.

An example of a place where multiple related companies work together on their side business instead of their core business is an airport. Here, the airport itself, airlines, and services at the airport all have their own core business, but they collaborate on a common theme. The common theme that Levrouw and Van Lubek identified among the companies involved in their project is predictive maintenance. This is about maintenance that should occur at companies at some point, but where it is uncertain when the maintenance is required. Levrouw and Van Lubek found that this is relevant to all companies involved but none of their core businesses, allowing them to work together.

To conclude, the PACES model has not been tested yet. It might therefore be challenging to fully apply the model to the case of SynCellEU. Still, we believe that the challenges identified to develop the PACES model, as well as the emotional driver of suspense, are relevant to the case because of the aforementioned reasons. We therefore developed an intervention in the form of a brainstorm session. The brainstorm session is based on the PACES model, where we focus on the first three "P's" of the model and the emotional driver of suspense. The first three "P's" of the model are "perceive", "perspective", and "predict", and are about opening up to sketch future scenarios with a certain predictive quality (Subsection 11.5.6). Part of the intervention will be to form uncertainty-excitement links, where the idea is to make use of excitement to overcome uncertainties. This fits to the advice from the European Commission to focus on opportunities instead of barriers and to create trust while forming university-industry collaborations [166] (Section). The The brainstorm session was initially planned during the BaSyC Winter Meeting of 2022, but due to COVID-19, this meeting has been postponed to spring later this year. The brainstorm session is therefore incorporated in the advice (Chapter 15). Since we were not able to perform the brainstorm session, it should still be validated if it is a useful intervention for SynCellEU.

A summary of this chapter is provided in Figure 13.1.

Summary of Chapter 13: Application of a model to the case

- Since SynCellEU currently comprises mainly of universities and research institutes, we would ideally apply a model with an academic perspective to the case. However, the academic models were not found suitable to apply to the case.
- Descriptive models were valuable to relate to the case, but are not found useful to further apply to the case.
- Analytical models will become useful to assess the performance of the innovation ecosystem once it has been formed.
- At this stage, it is useful for SynCellEU to apply a strategic model. The outcomes of this model can be incorporated in their vision to build an innovation ecosystem.
- The five challenges on which the strategic PACES model was based, as well as its underlying emotional driver of suspense, fit to the case of SynCellEU.
- Due to the long time required to form university-industry collaborations, the PACES model has not been tested yet by its developers.
- We developed an intervention based on the PACES model, where synthetic cell applications are specified and uncertainties are overcome through human excitement for novelty (Section 15.1). The effectiveness of the intervention should still be validated.

Figure 13.1 Summary of Chapter 13: Application of a model to the case.

14 Discussion

14.1 The variety in innovation ecosystem descriptions

In this study, we investigated multiple definitions and models describing innovation ecosystems. Through this analysis, we found a great variety in descriptions of the concept in literature. In Subsection 7.1.1, we discussed theoretical definitions of innovation ecosystems. Gomes et al. proposed a definition where co-production involving a central firm is key [117]. Here, it was postulated that competition and value capture are characteristic of business ecosystems, whereas collaboration and value creation are typical for innovation ecosystems. According to Adner and Kapoor, value capture and value creation should not be seen as two isolated entities [121]. Instead, they believe that value capture follows upon value creation in ecosystems. This linear way of thinking suggests that, in their vision to form an innovation ecosystem, SynCellEU should first create value through the development of applications, after which their efforts can bring economic prosperity through value capture.

Contrary to Gomes et al., Granstrand and Holgersson believe that innovation ecosystems literature unjustly focused too much on collaboration and value creation [120]. They believe that competition is part of innovation ecosystems as well, since the replacement of artefacts and resources plays an important role in both natural and artificial ecosystems. Contrary to the results of aforementioned reviews, we found that competition is considered to be relevant for innovation ecosystems in the majority of articles analysed in the systematic literature review (Section 11.9). Our findings thus correspond with the definition of Granstrand and Holgersson, where competition is seen as an integral part of innovation ecosystems. A possible explanation for this difference in findings is that our systematic literature review had a more specific scope. Next to this, our findings generally contained more recent articles, especially compared to Gomes et al., who analysed articles from 1993 to 2016 [117]. We hypothesise that competition is more relevant in the emerging research direction of university-industry collaborations in innovation ecosystems. In the case of SynCellEU, we found that competition between companies does not yet play a role, but that it can become relevant in the future (Section 9.4). The systematic literature review showed that companies can gain competitive advantage by joining a network (Section 11.9). Potentially, SynCellEU can utilise this idea to attract companies to their innovation ecosystem.

In the theoretical background of this study, Carayannis and Campbell are the only ones who specifically include universities in their description of an innovation ecosystem. However, they remain largely theoretical in their descriptions on the role of universities in innovation ecosystems, and how they should collaborate with other stakeholders. In the context of the quadruple helix model, it appears to be even more challenging to decipher the role of society in innovation ecosystems. In the systematic literature review (Chapter 11) and the chapter on the implementation of RRI elements (Chapter 12), we found some examples of collaborations that are relevant within a quadruple helix context, but practical examples remain limited. Indeed, Miller et al. pointed out that empirical evidence for quadruple helix implementations has only started to emerge in the previous decade [206].

Next to studying definitions of innovation ecosystems, we performed a systematic literature review on university-industry collaborations in innovation ecosystems. In this way, we showed the great variety that exists among innovation ecosystems, which is valuable for both SynCellEU and the research field in general. This does not mean that, in the case of SynCellEU, they should retain to a single definition of an innovation ecosystem and turn all their efforts towards complying to this definition. For them, the overview merely serves as handles they can use in the process of forming an innovation ecosystem,

and to get an idea of where they could head. As followed from the analysis, the formation of an innovation ecosystem is a dynamic process, and should be subjected to change based on insights that SynCellEU obtains along the way.

To classify the models identified in the systematic literature review, we developed a Model Classification Coordinate System (MCCS). With this tool, innovation ecosystem models were classified based on their focus on innovation, collaboration, academia, and industry. Next to this, models were categorised as either being strategic, descriptive, or analytical. Through the MCCS, we were able to create an overview of the vast amount of models that exist on university-industry collaborations in innovation ecosystems. The tool can also be used in the future to classify related models. The MCCS did not directly support us in selecting a model to apply to the case of SynCellEU, since no model with an academic perspective was found useful to be applied on the case (Section 13.1). However, it did allow us to select the PACES model on which we based our intervention (Section 15.1). We validated that the perspective of the PACES model fits to the case (Section 13.2). Since we were not able to perform the intervention, it should still be validated if it is a useful approach for SynCellEU to further specify synthetic cell applications.

14.2 Presenting the synthetic cell as a tool

We described opposing views on possible directions for applications in Section 10.3. SynCellEU identified three potential application fields: circular economy, medicine, and high-tech materials (Figure 10.2). The suggestions made by Bovenberg largely overlap with these application fields, especially with the latter two. Lohle, however, believes that SynCellEU should focus on a single application field. Next to this, he believes that the applications should not be presented as originating from a synthetic cell, since this is too fundamental. According to him, astrophysics is the only field that succeeded in a continuous focus on fundamental research while safeguarding funding. Although we agree with this latter point, we do not think that a synthetic cell is necessarily too fundamental. Instead, we think that a synthetic cell should be presented more as a tool that can contribute to grand challenges in the future, rather than a way to better understand life. While consulting the website of SynCellEU during the finishing stage of this study, we found that they already started to present the synthetic cell more as a tool: they added a slogan to their logo saying *"Nature is our next technology"*, and in their mission statement, they wrote that they aim to develop "100% green, cell-made materials and compounds" ([114], accessed on 15 January 2022).

By presenting the synthetic cell as a tool, it can be compared with the quantum computer and quantum internet (Section 10.2). Similar to the synthetic cell, these technologies stem from fundamental research, but are currently developed as applications at TU Delft. In the development of these quantum applications, TU Delft collaborates with the municipality of Delft and multiple companies [207], thereby adhering to the triple helix framework (Subsection 7.1.2). Next to Microsoft and Intel, a large part of the collaborating companies are start-ups and scale-ups, who contribute to the development of the quantum industry as pointed out by Lohle (Section 10.2). To realise synthetic cell applications in the future, a new industry should be built up as well. Similar to the quantum industry, it would be advantageous to have support from both large existing companies as well as more specialised start-ups in this case. To shorten the link between academia and industry, it could be beneficial to directly involve company employees in synthetic cell consortia on the longer term. Here, it should first be ensured that university and company employees get to know each other intensively, and that they know what they can expect from each other. Next to the development of a synthetic cell industry, company employees could assist in activities focused on marketing and branding.

14.3 Creating local foundations for a European innovation ecosystem

In Section 11.6, we described the diversity of scales on which innovation ecosystem operate. Innovation ecosystems were described on an organisational, regional, or national level. None of the identified articles described an innovation ecosystem as a transnational entity, although Schiuma and Carlucci mentioned that it can be globally branched through interrelated networks [149]. Other work on business and innovation ecosystems does consider the concept on a global scale, but this is mainly in the

context of business, without necessarily involving academia [208]. When relating this to the case of SynCellEU, it could thus be argued that the international innovation ecosystem they aspire is a collection of multiple regional innovation ecosystems in reality. This is not only a discussion about the definition of an innovation ecosystem, but it could have an effect on the actual successful implementation of it as well. If SynCellEU is focusing too much on international collaboration without properly having formed the regional synthetic cell hubs, the formation of a European innovation ecosystem might be impeded by the lack of strong local foundations.

To determine if SynCellEU should focus on local synthetic cell hubs, next to the European innovation ecosystem, it should be considered if local innovation ecosystems are beneficial in the development of synthetic cell products. For low-tech industries, it is clear that localising the innovation ecosystem is advantageous in terms of reducing supply chain costs [167]. For high-tech industries, however, it is less apparent if the level of knowledge and resource sharing is dependent on geographical location. In an article studying the biotech industry, it was found that regional clustering of related organisations is beneficial in terms of innovation ecosystem growth, since it provides start-ups with the resources they require to grow [209]. Zucker et al. [210] described that for new industries emerging from radical scientific findings, “proficiency in the new area requires the hands-on training from one of the technology’s pioneers” [209]. In this case, it is thus beneficial to locate start-ups close to a university that can provide this training. Geographical proximity of related organisations can also strengthen the local innovation ecosystem through knowledge spill-over, where ideas are shared between individuals. A study investigating the influence of knowledge spill-overs on the metropolitan region of Berlin found that over ten thousand jobs were formed through academic spin-offs [211]. Furthermore, triple helix literature showed that it can be advantageous for companies to be located close to universities or other research organisations [212].

Arguments in favour of locally clustering innovation ecosystems were also provided by Bandera and Thomas, in terms of easy access to resources and increased availability of social capital [167] (Subsection 11.5.8). However, from their model on start-up survival, it followed that the mere presence of social capital is not sufficient. Instead, organisations should genuinely make use of social capital to drive innovation. This corresponds with the analysis of Singh, who argues that knowledge diffusion is especially apparent if the actors within a region have close ties [213]. To increase the use of social capital, differences in social structures among organisation should be taken into account as described in Section 11.10. Approaches to overcome these differences could for example focus on joint seminars or talent exchange programmes, where both academia and industry are involved (Section 13.2). Part of the identified studies also highlighted the importance of informal communication for innovation ecosystem development (Section 11.11)

Although many sources confirm the advantage of locally clustering innovation ecosystems, especially in the context of start-up formation, there is no general consensus on this issue. Letaifa and Rebeau, for example, describe that the fear for competition may hamper social capital utilisation in dense ecosystems [214]. In fact, they found that companies who fear local competition prefer to collaborate with foreign companies, “as [these] partners do not feel threatened in their local markets” [214]. However, this was found to be especially relevant for ecosystems with a high level of rivalry, which is not yet the case for SynCellEU (Section 9.4). Stuart and Sorenson found that distant collaborations become more relevant as a new industry grows, since the networks of this new industry expand through international conferences and industry associations [209].

When relating this discussion to the case of SynCellEU, it suggests to first strengthen the local synthetic cell hubs, that can later be combined into a European innovation ecosystem. Although we believe that this is beneficial in terms of creating an industry for synthetic cells, we believe that SynCellEU should continue to develop academic collaborations on a European level at this point. Synthetic cell research has a highly international character, and through European collaboration, the realisation of a synthetic cell can be accelerated. These international communication channels could already be used to share knowledge and experiences on the formation of local synthetic cell hubs. In this way, the foundation for a European innovation ecosystem will be constructed in parallel.

14.4 Fostering an entrepreneurial culture

To ensure the formation of local synthetic cell hubs, it should thus be ensured that social capital is utilised. In the case of TU Delft, there are organisations that can assist in forming collaborations with existing companies, or that promote the formation of start-ups (e.g. the I&I Centre, Delft Enterprises, Yes!Delft, Section 10.4). However, it is uncertain if SynCellEU is ready to make use of this social capital once the TRL of synthetic cell research starts to climb. Bovenberg, for example, indicated that an entrepreneurial culture is less apparent in Europe compared to other parts in the world (Section 10.4), and Dogterom and Usai mentioned no plans to promote the formation of synthetic cell related start-ups. It is possible that they actually do have these plans, but that they were not brought up in the interviews. Another possibility is that they are not yet concerned with this, since synthetic cell applications are still far away. Still, we believe that it is important to start fostering an entrepreneurial culture at this point, to facilitate the formation of start-ups once the time is there.

The importance of promoting an entrepreneurial culture for innovation ecosystem development was also recognised by the systematic literature review (Section 11.8). In some articles, entrepreneurship was promoted among university students, for example by involving external organisations in the curriculum, or by organising entire study programmes focused on bioentrepreneurship. For Nanobiology, the study programme most closely related to synthetic cell research at TU Delft, courses teaching entrepreneurial skills are not included in the curriculum [215, 216]. However, Bachelor students have the possibility to follow a minor of 30 ECTS focused on a different subject, for example the minor in MedTech-Based Entrepreneurship [217]. Master students can follow a free elective focused on entrepreneurship, but this possibility is not actively promoted. PhD students at TU Delft have to follow courses at the Graduate School next to performing research [218]. To check if this programme offers entrepreneurial courses, we consulted two TU Delft PhD students. They found a single workshop on "turning your thesis into a business", but no elaborate offer of courses to train entrepreneurial skills. There are more extensive courses offered by the Delft Centre for Entrepreneurship [186], but it is unknown if this programme is sufficiently promoted among PhD students performing synthetic cell related research.

Next to offering entrepreneurial education, academic support in the formation of start-ups or spin-offs was mentioned to be important by Bovenberg (Section 10.4) as well as by Sinell et al. (Section 11.8). To elaborate on this, Rasmussen et al. showed that departmental support is considered to be more important than institutional support in terms of spin-off formation [219]. They stated that “at a local level the value of [available] resources for the early exploration of the commercial potential of research appears crucial but unless supported by department management will remain less effective” [219]. Since the majority of studies on new venture creation focus on the university level, they suggest that more research into the role of the department is required.

In Section 11.8, we mentioned that in some countries, national funding programmes exist to promote the formation of spin-offs. We found no such funding mechanism for the Netherlands, but did find an investment fund that specifically supports entrepreneurs from Zuid-Holland, the province where TU Delft is located. This investment fund, called UNIIQ, was set up in 2016 by TU Delft, Erasmus Medical Centre (Rotterdam), and the University of Leiden to promote regional innovation development [220]. Next to this, we described that scientists often have a higher chance to obtain research grants if their work has the potential to be transferred (Section 11.8). In case of SynCellEU, this would mean that they could continue their fundamental research with this type of funding, while also promoting technology transfer. In Section 10.4, we described that academic transfer activities can be promoted by offering career benefits to scientists, but that no such advantages are offered by the I&I Centre at TU Delft. Possibly, technology transfer can be further promoted by offering a selection of these career benefits.

14.5 A future shift in the European lobby

Next to promoting the formation of start-ups and spin-offs, we believe it is important to identify potentially interesting companies that already exist. The intervention proposed in the advice (Section 15.1) could serve as a starting point to identify these companies. When synthetic cell application fields are

further specified, a list with potential industry partners could be formalised. A specification of these applications would be beneficial for the lobby in Brussels as well, where European parliamentarians ask for concrete examples of future synthetic cell products (Section 10.1).

We believe that SynCellEU should continue their lobby for a medium-sized research grant in Brussels (Section 9.3), since a European collaboration is likely to accelerate the formation of a synthetic cell. The following arguments can be used in this lobby: Europe has a strong foundation in bottom-up biology, Europe could lose its head starts to other parts in the world when it does not act, and synthetic cells can result in revolutionary applications. At the same time, we believe that lobbying activities should increasingly focus on the realisation of European transfer opportunities. These will become relevant once local synthetic cell hubs have been formed, and when their assets will be bundled in a European innovation ecosystem.

Back in 2010, the European Commission already indicated the need to promote knowledge transfer in research organisations [221]. In 2016, the European Commission stated that technology transfer in Europe "shows similarities with an emerging industry: many valuable product ideas, a highly fragmented landscape, a lack of critical mass, and wide disparities in terms of performances and developing practices" [222]. Next to this, a study conducted in the same year found that the majority of European universities and companies were not involved in university-industry collaborations [196]. However, it was also mentioned by the European Commission that drastic changes in terms of knowledge and technology transfer were expected, due to an increasing amount of incentives for transfer activities [222].

Indeed, a lot has changed at TU Delft since that time in terms of technology transfer (Section 10.4). A further increase in technology transfer could for example be facilitated by offering scientists the ability to adopt hybrid roles, allowing them to perform both academic and entrepreneurial activities. In an extensive study by Jain et al., they found that academic entrepreneurs are generally able to see their two different roles as separate entities, thereby safeguarding their academic identity [223]. This can be used as an argument against criticism on the Bayh-Dole Act, where it was argued that an academic focus on transfer activities might jeopardise a proper execution of the scientific method (Section 13.2). Another type of incentive to promote European technology transfer is funding. In 2021, the European Commission pended a call for a grant focused on innovations with a TRL of 4-5 [224]. In the future, a similar type of funding could become interesting for SynCellEU, especially since the European Commission desires international collaborations [221].

14.6 The implementation of RRI elements

Through our analysis, we found that public engagement is becoming increasingly relevant for innovation ecosystems, especially in the context of the quadruple helix framework (Subsection 7.1.2). However, clear and concrete examples of public engagement remain scarce in literature. We discussed the case of BrisSynBio, a synthetic biology research centre where public engagement was internalised from the start (Subsection 12.2.2). Since SynCellEU is currently in its starting phase, this offers opportunities to implement public engagement and other RRI elements from the beginning of the collaboration. One challenge here, however, is that multiple stakeholders will be involved in the innovation ecosystem SynCellEU aims to form, whereas in the case of BrisSynBio, it was a homogeneous group of researchers that were enthusiastic about public engagement. The differences in perspectives and values among the potential stakeholders of the synthetic cell innovation ecosystem should therefore be taken into account. Here, strategies focusing on increasing social proximity will likely contribute to forming a common vision.

From the case study on BrisSynBio, it followed that starting PhD students are generally more interested in RRI related activities than older scientists, possibly because of an early exposure to RRI elements in the careers of the initial group. In the case of SynCellEU, it could therefore be beneficial to make RRI related opportunities more widely available to Bachelor, Master and PhD students. This could for example be done by organising seminars on RRI elements, where its related concepts are introduced to students in an engaging way, focusing on relevant, real-world problems. Next to this, we believe

there should be a central point of contact for people interested in performing RRI related activities, to avoid cases where enthusiasm is left unused (Section 12.2).

Similar to fostering an entrepreneurial culture, the implementation of RRI elements could be promoted by allowing hybrid roles. At TU Delft, it would for example be an option to let PhD students earn credits for the Graduate School by organising a public engagement event. Here, it should be ensured that there is proper communication between people focusing on RRI elements and people focusing on entrepreneurship, such that the wishes of society can be integrated with synthetic cell products. This could for example be facilitated by activities similar to the intervention described in Section 15.1, with the aim that people integrate shared insights in their daily work activities. Another way to integrate societal wishes with product commercialisation is through collaborations between TTOs and university organisations concerned with public relations (PR). According to Sinell et al., activities organised by PR, such as open lectures and public engagement events "bring findings and inventions nearer to the public, suggesting the need for closer collaboration and possibly even a merger between PR and transfer offices" [176]. At TU Delft, the I&I Centre is increasingly taking the wishes of society into account (Section 10.4), and is responsible for both technology transfer and PR. In this way, it allows for the integration of public engagement and commercialisation of synthetic cell products in the future.

Next to public engagement, we also studied the RRI element of open science & open innovation. We found that open science does not necessarily lead to open innovation, since innovation often requires at least to some degree IP protection. This protection is for example required to stimulate investments in the innovation. To sooth the potential tension between this RRI element and IP protection, the level of IP should be properly balanced. Here, one can for example only protect the foreground knowledge that is required to develop a specific innovation, but make the background knowledge freely available. This background knowledge can then be used by other scientists and entrepreneurs for future studies and innovations [201]. A disadvantage of IP protection is that it can slow down the innovation process. Although Jacobs indicated that, in general, a patent application only takes a few weeks to a few months (Section 12.3), this can take up to eighteen months in the Netherlands [225]. Furthermore, a European patent application takes on average three to five years [226]. When relating these findings to SynCellEU, they should critically think about the degree to which they want to protect their findings, and on what level they want to apply for patents. The long duration of a European patent application makes it more attractive to first protect findings on a national level, but applications developed in a European innovation ecosystem might require European protection. The patents office of the I&I Centre at TU Delft can assist SynCellEU in making these considerations.

We studied the RRI element of sustainability as well. Here, we found that SynCellEU can possibly play an important role in the development of sustainable innovations, for example in terms of optimising production processes, or by promoting the realisation of sustainable products by SMEs through university-industry collaborations (Section 12.4). We also believe that sustainability will be of relevance in the expansion of the synthetic cell network. Although we postulate that it is beneficial for SynCellEU to first strengthen the local innovation ecosystems, their long term ambition is to form a European synthetic cell network. With the expansion of the network, physical distances between individual partners of the ecosystem increase. Here, it should be ensured that the parties across Europe are able to properly interact with each other. As Shvetsova and Lee put it, "it is a well-known fact that good infrastructure lays the foundation for a vibrant ecosystem" [152]. When relating this to the RRI element of sustainability, it could be argued that SynCellEU would benefit from an improved European train infrastructure. In Section 13.2, we described that informal communication can contribute to the development of the innovation ecosystem. Although informal communication is still possible in a digital way, it is not promoted, "since [communication technologies] lack real and perceived presence of others, as well as a shared social setting" [227]. We therefore believe that SynCellEU would benefit from improved rail connections across Europe, to ensure meaningful interactions in a sustainable way.

Finally, we discussed articles describing approaches to form innovation ecosystems in regions with less economic prosperity, for example the research agenda proposed by Schiuma and Carlucci [149] (Subsection 11.5.1). In her interview, Dogterom indicated that SynCellEU aims to develop innovations that are not only beneficial for rich Western countries (Chapter 12). Although we did not discuss include

this RRI element of global inclusion in our descriptive literature review, we believe that the discussed models can contribute to the development of innovation ecosystems in less-developed areas. Here, the social heterogeneity of relevant stakeholders should be taken into account, to avoid attempts where thriving innovation ecosystems such as Silicon Valley are unsuccessfully copied (Section 11.10).

14.7 Research methods

The first two sub-questions of this study, which addressed the vision and innovative context of SynCellEU, were answered by collecting both primary and secondary data. The primary data was obtained by performing semi-structured interviews, whereas the secondary data was collected by studying web pages, online news articles, and the BaSyC application grant. We chose to conduct semi-structured interviews, since this method allowed us to add interview questions based on the insights obtained during the interviews. Furthermore, semi-structured interviews generally result in a high level of validity, because they provide deep insights in the thoughts, feelings, and ideas of the interviewees [228]. One of the research limitations of our approach is that the interview sample was relatively small. Bovenberg, for example, was the only person we interviewed from DSM, and his ideas might not reflect the vision of the company as a whole. Still, we aimed to ensure validity by finding common patterns among the interview results, and by relating the interview results with insights from literature (Section 8.2).

Results that are obtained with semi-structured interviews depend on the questions that are asked. This means that other insights were possibly obtained if our study was conducted by other researchers. Still, we aimed to maximise reliability of the approach by attaching our interview protocols in Appendix E. Another limitation of the chosen approach is that we mainly focused on TU Delft, and not on the other universities and research institutes involved in SynCellEU. Next to this, we mainly focused on managerial aspects of the case by interviewing people holding high positions. The ideas of these professional do not necessarily reflect the opinions of for example PhD students and postdocs, people who will also play an important role in the formation of a European innovation ecosystem. For future research, it would therefore be interesting to study the other universities and research institutes involved in SynCellEU, as well as the views and ideas of other relevant people. To obtain a broader general overview of the case, instead of the in-depth case study we performed, one could make use of surveys to collect data from a larger number of participants.

We performed a systematic literature review on models describing or analysing university-industry collaborations in innovation ecosystems. Here, we ensured reliability by providing our search terms and by explaining our article selection procedure (Section 8.2). The validity of our approach was safeguarded by constructing a code-to-article matrix (Appendix G), where we extensively assessed overlapping themes between the identified articles. One of the limitations of the code-to-article matrix is that we did not clearly define a cut-off value between codes either being "discussed extensively" or "discussed moderately". However, we mainly wanted to identify if a code was discussed, mentioned, or not mentioned, making this distinction less relevant. Another limitation of our approach is that we specifically focused on models describing university-industry collaborations in our systematic literature review. Although the models allowed us to extract valuable insights, the literature search possibly excluded other interesting articles that did not contain a model.

From the systematic literature review, we extracted the following RRI elements: public engagement, open science & open innovation, and sustainability (Section 12.1). By relating the RRI elements to the six RRI dimensions described by Kwee et al. [137], we confirmed that they are indeed concepts described by RRI. Finally, we verified that the extracted RRI elements are relevant to the case by relating them to the interview transcripts. A limitation of this approach is that we did not cover the entire spectrum of RRI, and other RRI elements might be relevant to the case as well. For the descriptive literature review on the extracted RRI elements, we included the articles from the systematic literature review that discussed these specific RRI elements. Although they provided us with interesting insights, their scope deviated a lot from this study in some cases, making the articles less interesting in the context of SynCellEU. For future research, it would be interesting to perform a more directed literature search on specific RRI elements an their relationship with the natural sciences. In this way, we could get deeper insights in approaches to implement RRI elements in a synthetic cell innovation ecosystem.

In Figure 14.1, a summary of the discussion is provided.

Summary of Chapter 14: Discussion

- We found that competition is part of innovation ecosystems, which corresponds to the definition of an innovation evosystem by Granstrand and Holgersson [117].
- With our systemtic literature review, we showed the great variety in innovation ecosytem descriptions. Full practical examples of quadruple helix model implementations in the context of innovation ecosystems remain scarce.
- We developed a Model Classification Coordinate System (MCCS). We used this tool to select a model on which we based the intervention. The MCCS can be used in the future to classify models on university-industry collaborations in innovation ecosystems.
- The synthetic cell should be presented as a tool that can contribute to grand challenges in the future, rather than a way to better understand life.
- The realisation of synthetic cell applications requires the development of a new industry, where the involvement of large existing companies as well as start-ups is preferred.
- SynCellEU should first strengthen the local synthetic cell hubs, that can later be combined into a Europen innovation ecosystem.
- SynCellEU should invest in an entrepreneurial culture to ensure the utilisation of social capital once synthetic cell applications start to emerge.
- A further specificication of synthetic cell applications is required to identify potential industry partners. This will also increase the chances to obtain funding.
- SynCellEU should continute its lobby at the EU for a medium-sized research grant. The focus of the lobby should increasingly focus on European transfer possibilities.
- Public engagement can be promoted by making RRI-related activities available to all university students and employees, and by allowing hybrid role identities. There should be a central point of contact for people who are interested in RRI-related activities.
- To allow for co-production, SynCellEU should critically assess to which degree they want to protect their findings.
- SynCellEU can play an important role in the development of sustainable innovations. To integrate sustainability with the European innovation ecosystem, SynCellEU would benefit from an improved European train infrastructure.
- The methods to safeguard validity and reliability of this study as well as the research limitations can be found in Section 14.7.

Figure 14.1 Summary of Chapter 14: Discussion.

15
Advice

Following upon the insights obtained in this study, and the points we discussed in Chapter 14, we would like to propose an advice focused on SynCellEU's aim to build an innovation ecosystem. In this advice, we will first propose an intervention to further specify synthetic cell applications. This could be valuable both in terms of identifying potential industry partners and to come up with possible business ideas for start-ups. It should still be validated if the intervention is indeed a useful approach for SynCellEU to specify synthetic cell applications.

After presenting the intervention, we will sketch two scenarios through which SynCellEU can build a synthetic cell innovation ecosystem. These scenarios are focused on the promotion of bottom-up activities, and the execution of top-down activities, respectively. We believe that SynCellEU is currently mainly invested in top-down activities. By sketching a scenario where SynCellEU promotes the execution of bottom-up initiatives, we thus provide a distinctive perspective with our advice. To build a sustainable synthetic cell innovation ecosystem, we believe that both scenarios should be combined with each other in the strategy of SynCellEU, where the two scenarios can stimulate each other in a synergistic manner.

Our discussion showed that new industries are often formed locally, and only expand once the industry grows mature (Section 14.3). We therefore suggest to first invest in local synthetic cell hubs across Europe, that can later be combined into a European innovation ecosystem. Without the development of these local synthetic cell hubs, the European innovation ecosystem might lack a strong foundation that it requires to ensure strong and sustainable collaborations. Once the local synthetic cell hubs have been formed, specific actions are required to prevent them from becoming isolated in the network. Scenario I will contribute to the formation of local synthetic cell hubs, and Scenario II will play a role both on a local and on a European scale.

15.1 An intervention to specify synthetic cell applications

Background

Potential application fields of synthetic cells were already identified in an earlier brainstorm session. The results of this brainstorm session are visible in Figure 10.2. A further specification of the applications in these fields is required to identify potential industry partners, and to possibly formulate business ideas for start-ups. We will therefore present an additional brainstorm session in this intervention. The participants of this intervention are PhD students, postdocs, and PIs involved in synthetic cell research, for example from the BaSyC project. If possible, it would be interesting to involve industry partners as well. The brainstorm session should be moderated by someone with extensive experience with brainstorming and/or someone with a background in communication sciences, for example a Master's student or employee from the Science Education and Communication (SEC) department at TU Delft.

Goal

The goal of the intervention is to further work out the synthetic cell products identified in the earlier brainstorm session. This will be done based on the feeling of "suspense" – the uncertainty and ex-

citement one feels about the future. Suspense has been identified as an emotional driver for many university-industry collaborations, where it is aimed to make use of excitement to overcome uncertainties [157]. The results of the brainstorm workshop can be used to make the strategy in terms of university-industry collaborations more concrete, possibly in a follow-up session.

Format

The workshop takes ~1.5 hour and requires ten to twenty participants in two to four groups. The number of groups depends on an online or offline organisation of the session, where it is important that all group members are able to actively participate in the session. In case of a physical session, the room acoustics should be taken into account in determining the number of groups. Each group is assigned one of the pre-identified synthetic cell products (for example smart biosensors, self-healing responsive materials or artificial food from the high-tech materials category, see Figure 10.2). The brainstorming takes place on the brainstorm sheet (Figure 15.1). Since it is likely that many of the participants have little experience with brainstorming, it is important that sufficient time is taken for the session, and that a short break is included. The different parts of the session and a time indication of each part are displayed below:

1. **Introduction (10 min.)** - Explanation about this thesis project and the brainstorm workshop.

2. **Brainstorming phase (30 min.)** - Brainstorming about the different synthetic cell products or product categories. Each group should come up with as many ideas related to their synthetic cell product as possible. In this round, there are no boundaries yet and everything is possible, allowing for out-of-the-box ideas to emerge. Participants can draw and write ideas on the brainstorm sheet using post its. Icebreaker questions or assignments will be visible on the brainstorm sheet to start the discussion. Below, some examples of icebreaker assignments are provided:

   "*If I could make any type of synthetic cell, I would make a ... cell, because ...*"

   "*If I could make any type of material with synthetic cells, I would make ..., because ...*"

   "*If I could make any type of food with synthetic cells, I would make ..., because ...*"

   "*The thing that excites me the most about synthetic cell research is ..., because ...*"

   "*When I explain my research to laymen, I tell them my research is about ...*"

3. **Reflection phase (10 min.)** – Participants think for themselves what excites them about the synthetic cell products (e.g. what kind of future do they envision, how do they think that their (research) activities/interests could contribute to this), and what makes them uncertain (e.g. what hurdles do they foresee along the road, what makes people personally uncertain).

4. **Break (10 min.)**

5. **Construction phase (15 min.)** – Participants share their excitements and uncertainties, and discuss how to make use of (each other's) excitements to overcome uncertainties or to come up with new ideas. The participants write down suspense links on the brainstorm sheet, connecting uncertainties to excitement. A few examples of such a suspense links are described below:

   One group is discussing the development of artificial meat. A participant of this group is vegetarian for environmental reasons, but is excited about eating meat again when it is grown in a lab.

At the same time, they are uncertain about what society might think of producing meat in a lab. Another participant is excited about making science communication animations, and would like to make a video explaining the safety of artificial meat.

One group is discussing how their research could be used to develop smart biosensors. One of the PhD students in the group has developed an approach to measure a complex chemical reaction, and is considering to develop a commercial tool that could measure this reaction. However, they are uncertain about how to approach this. One of the PIs of the group has connections with a business incubator, and is excited about turning this promising idea into an application.

One group is discussing the formation of university-industry collaborations in the field of tissue regeneration. One PI in the group is performing research on this subject, and would like to apply their knowledge through a collaboration with a company, but is uncertain which companies to approach. One PhD student of the group did an internship at a related company, and is excited to share the contact details of their supervisor.

6. **Evaluation (15 min.)** - Groups share their most interesting findings (using the brainstorm sheet) and the moderator wraps up the session.

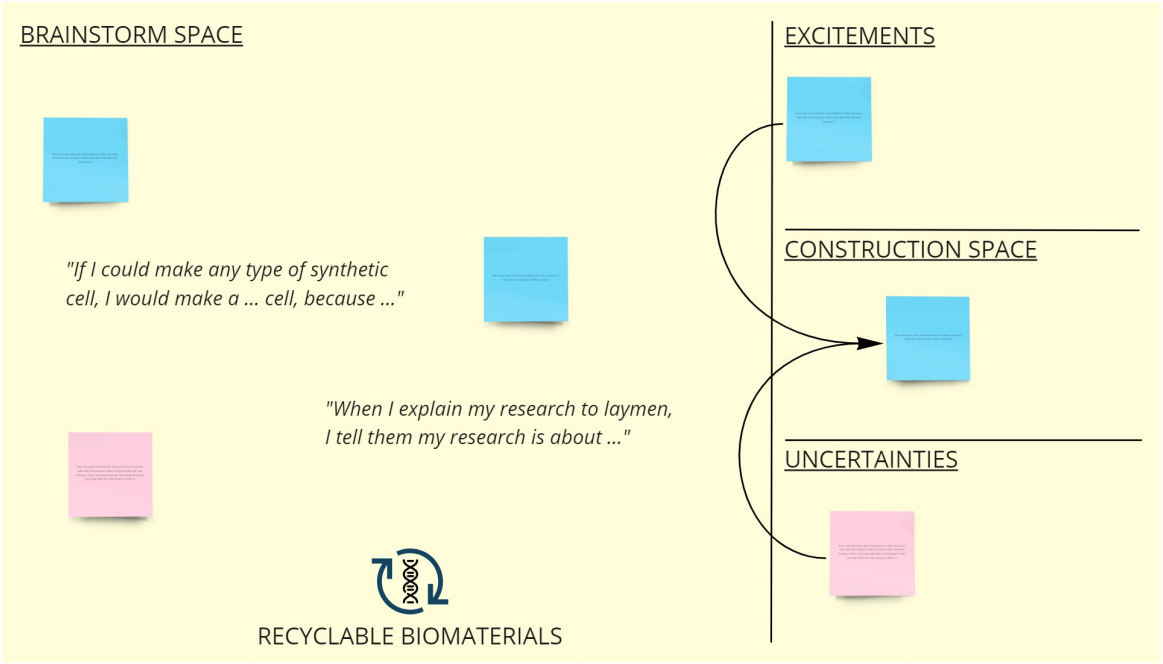


Figure 15.1 Example of the brainstorm sheet. Each group is assigned with their own synthetic cell product, in this case "recyclable biomaterials". Participants can draw, write text, and make use of post-its. On the brainstorm sheet, icebreaker assignments are displayed to start the discussion. All ideas from the brainstorming phase are collected in the "brainstorm space". The ideas from the reflection phase are placed at "excitements" and "uncertainties". Uncertainty-excitement links are formed in the "construction space". Figure made with Miro.

15.2 Scenario I: Promoting the organisation of bottom-up activities

The first scenario consists of action points that, upon execution by SynCellEU at their involved universities and research institutes, should lead to the promotion of bottom-up activities. The action points are aimed at fostering an entrepreneurial culture and encouraging the performance of RRI related activities. Potential bottom-up activities that could stem from this scenario are public engagement events and the formation of start-ups. In this way, the first scenario contributes to the formation of local synthetic cell hubs.

15.2.1 Creating strong innovative foundations

A European synthetic cell innovation ecosystem requires strong foundations in the form of local synthetic cell hubs. These pioneering communities can later be bundled into a strong and sustainable European innovation ecosystem. To promote the execution of entrepreneurial bottom-up activities, local initiatives should mainly focus on fostering an entrepreneurial culture, thereby increasing innovative potential. We recommend to organise this with the following action points, where they are listed from highest to lower priority:

- Offer (PhD) students the possibility to follow courses focused on training entrepreneurial skills. At TU Delft, Nanobiology Master students should have the option to follow an additional course on entrepreneurship next to their free elective. Next to this, the courses offered by the Delft Centre for Entrepreneurship (Section 14.4) should be actively promoted among PhD students, and it should be possible to follow them for the Graduate School.
- Postdocs and principal investigators should be allowed to adopt hybrid roles (Section 14.5), thereby focusing on both research and transfer activities.
- Ensure effective communication between people implementing RRI elements and people working on technology transfer, thereby taking the wishes of society into account in product commercialisation.
- Organise support for academic entrepreneurship at the departmental level, since this was found to be more effective [219].
- Organise exchanges with research departments where academic entrepreneurship is more actively executed.

We foresee that these action points will result in entrepreneurial bottom-up activities within 2-5 years.

15.2.2 Planting the seeds for sustainable growth

The implementation of RRI elements starts by introducing the elements in an accessible and engaging way to students and employees, thereby creating an RRI mindset. At the same time, it should be ensured that RRI related activities are available to everyone at the organisation. In this way, people who are intrinsically motivated and have affinity with the subject will stand up to execute the actual implementation of RRI elements. We recommend to facilitate this in the following way, where the action points are again listed from highest to lower priority:

- Offer opportunities for people interested in RRI related activities by appointing a central point of contact. This person is responsible for bringing interested people together.
- Organise seminars on RRI related themes at least once each quarter. Aim to show concrete real-world examples of how RRI elements are implemented in research or transfer activities. Invite both students and employees to these seminars. The central point of contact can also be promoted at these seminars.
- Allow PhD students to perform RRI related activities by offering rewards. In the case of TU Delft, PhD students could for example earn credits for the Graduate School by organising a public engagement event.

- Organise exchanges with universities and research institutes where certain RRI elements have been internalised effectively, such as public engagement at BrisSynBio (Subsection 12.2.2).

We foresee that these action points will result in the organisation of bottom-up RRI activities within 1-3 years.

15.3 Scenario II: Shaping the innovation ecosystem with top-down activities

The second scenario focuses on top-down activities. In this scenario, SynCellEU initially takes the lead as well, but increasingly explores how responsibilities can shift towards one or multiple industry partners. These industry partners will first collaborate with the local synthetic cell hubs, where the collaborations can later expand to a European level. The second scenario also comprises the lobbying activities in Brussels, where the scope of the lobby should increasingly focus on technology transfer possibilities next to the realisation of fundamental research. In this way, the second scenario contributes both to the development of local synthetic cell hubs and to the formation of a European innovation ecosystem.

15.3.1 Forming university-industry collaborations

SynCellEU should continue and expand the contact with existing companies to explore the industry potential of synthetic cells. They should keep an eye open for small companies, since these are likely to become increasingly interested in collaborations as they grow in size (Section 13.2). Next to this, potential synthetic cell applications should be further specified, and the utilisation of social capital (Subsection 11.5.8) should be increased. Here, the synthetic cell should be presented as a tool that can contribute to sustainable applications in the identified fields. The role of RRI in university-industry collaborations should be explored as well. Finally, SynCellEU should connect with the right parties once applications start to emerge. We propose to do this with the following action points, where they are again listed from highest to lower priority:

- Further specify synthetic cell applications through the intervention proposed in the beginning of this advice (Section 15.1), and formulate a list of potentially interesting companies based on the outcomes of the intervention.

- Interact with industry by organising joint seminars, talent exchange programmes, and/or company involvement in study curricula

- Discuss RRI elements with industrial contacts. These discussions could for example focus on how university-industry collaborations can contribute to the energy transition, or how inclusivity can be ensured in collaborations. Formulate concrete plans from these discussions and incorporate them in (joint) funding applications.

- Explore the possibilities of involving company employees in synthetic cell consortia. Next to the development of synthetic cell applications, company employees could possibly assist with marketing and branding activities.

- Have concrete plans ready to form university-industry collaborations that can be executed once there is a clearer view on potential synthetic cell applications.

- Keep an eye open for funding and support opportunities, and seize these opportunities once synthetic cell applications start to emerge. Examples relevant for TU Delft are the UNIIQ grant (Section 14.4) and organisations concerned with innovation and entrepreneurship (Section 10.4).

We foresee that these action points will result in formal university-industry collaborations within 3-7 years.

15.3.2 Bundling the local synthetic cell hubs into a European innovation ecosystem

Once the local synthetic cell hubs have formed strong foundations, it is time to connect them into a sustainable European innovation ecosystem. During the development of local synthetic cell hubs, European collaboration should already take place on an academic level, since this will likely accelerate the development of a synthetic cell. The academic European collaboration channels can also be used to share knowledge and experiences on building the local synthetic cell hubs, for example during international conferences. We recommend to organise the European innovation ecosystem with the following action points, where they are listed in chronological order of execution:

- Continue the European lobby for a medium-sized research grant. In this lobby, the potential to develop sustainable products from synthetic cells as well as the efforts to implement RRI elements in local synthetic cell hubs should be highlighted. Next to this, it should be stressed that Europe has a strong foundation in bottom-up biology, but that it might lose its head start to other parts in the world if it does not act.
- Increasingly shift the focus of synthetic cell conferences from a purely scientific debate to a more elaborate focus on commercial and societal issues, thereby attracting more industry partners and governmental organisations.
- When local synthetic cell hubs have been formed, their integration can be facilitated through international conferences, joint projects and exchange programmes.
- Once the European synthetic cell innovation ecosystem is formed, its performance can be assessed with the analytical models discussed in Chapter 11.

Together with the earlier actions focused on building local synthetic cell hubs, we foresee that these action points will result in an integrated European innovation ecosystem within 10-15 years.

16
Conclusion

In this project, we performed a case study on SynCellEU's vision to build an innovation ecosystem. We first studied the details of this vision by addressing the first sub-question:

1. What does the vision of SynCellEU to build an innovation ecosystem entail?

From the analysis of the website of SynCellEU, we found that they present themselves as a cutting-edge community. Compared to the Dutch research collaboration BaSyC, they are more focused on exploring potential synthetic cell applications. Through semi-structured interviews with the Programme Manager and one of the Promotors of SynCellEU, we found that the details of the future innovation ecosystem are unknown, since SynCellEU constantly has to adapt its strategy based on dynamic changes. However, they have the long-term vision to develop synthetic cell products through co-production. SynCellEU is currently forming the basis for collaborations with two approaches. Firstly, they are performing lobbying activities at the European Commission in Brussels. Here, they aim to put the synthetic cell on the agenda. Next to this, they promote the formation of a medium-sized research grant, to expand the research activities of BaSyC to a European level. Secondly, they are having conversations with companies on possible future directions of a synthetic cell. The current industrial contacts were formed both through pre-existing contacts and by actively approaching other companies.

To investigate what kind of companies SynCellEU is currently having conversations with, and to explore the innovative context of the case in general, we discussed the following sub-question:

2. What does the innovative context of SynCellEU look like?

Here, we found that the majority of current contacts exists of chemical and food & nutrition companies, possibly formed by pre-existing contacts of the chemical researchers involved in the collaboration. Next to this, there are some pharmaceutical and synthetic biology companies who have shown interest in synthetic cells. These companies have indicated that they are interested in the developments of synthetic cell research, but no formal collaborations have been formed yet.

SynCellEU already identified three possible application directions, focused on circular economy, medicine, and high-tech materials. These application fields largely overlap with the ideas of the industry expert we interviewed. The former Business Relations Manager of the faculty of Applied Sciences at TU Delft, however, believes that the innovation ecosystem should solely focus on the development of materials. Next to the former Business Relations manager of the aforementioned faculty, we also interviewed the person currently holding this position. Together, they gave us insights in the activities of the I&I Centre, the organisation responsible for technology transfer at TU Delft. Over the last eight years, their strategy changed towards increasingly including the wishes of society in product commercialisation.

Next to the I&I Centre, we described two other organisations present in the innovative environment of TU Delft: the business incubator YES!Delft, and a private organisation supporting spin-offs called Delft Enterprises. From the analysis on the innovative context of SynCellEU, it also followed that an entrepreneurial culture might be lacking in Europe. This could potentially hamper the foundation of spin-offs based on synthetic cell related research.

There are many definitions of the concept of innovation ecosystems. Next to this, the details of the innovation ecosystem that SynCellEU aspires are unclear. To understand how university-industry collaborations are formed within innovation ecosystems, we performed a systematic literature review addressing the following question:

3. What innovation and collaboration models best fit to the case of SynCellEU pursuing to build an innovation ecosystem?

Through this systematic literature review, we found a great variety of descriptive, strategic, and analytical models describing partnerships between universities and companies in innovation ecosystems. To create an overview of the identified models, and to classify them based on their perspective and focus, we developed a Model Classification Coordinate System (MCCS). From the analysis, we found that strategic models are the best fit to SynCellEU at this stage, since they are concerned with activities to form university-industry collaborations. Next to this, the descriptive models gave insights in the formation of other innovation ecosystems through case studies. Finally, we found that the analytical models will become more relevant as the synthetic cell innovation ecosystem grows. With these models, the performance of the network can be assessed. Through the systematic literature review and an additional descriptive literature review, we discussed the following sub-question:

4. In what way can RRI elements be implemented in the innovation ecosystem SynCellEU aims to build?

Here, we discussed the RRI elements of public engagement, open science & open innovation, and sustainability. Public engagement was extensively studied in the research centre BrisSynBio, where they succeeded in internalising this RRI element by implementing it from the start. They did this by making RRI related activities available to all university students and employees, which resulted in the exploration of RRI through case studies, workshops and a theatre play. We found that open science does not necessarily result in open innovation, and that IP protection should be properly balanced to facilitate both processes. The analysis on sustainability showed that SynCellEU can play an important role in the development of sustainable innovations, both in terms of optimising production processes and by assisting SMEs in their activities to bring sustainable products to the market. However, more in-depth case studies are required to further explore the role of the university in the development of sustainable applications.

The four sub-questions together addressed the main research question of this study:

In what way can SynCellEU lay the foundation for a sustainable innovation ecosystem?

Here, we concluded that the synthetic cell should be presented as a tool rather than an approach to understand life, especially in the context of the European collaboration. Applications of this tool can be further specified with the intervention we proposed, where it is aimed to overcome uncertainties by focusing on human excitement for novelty. By increasing the possibilities for technology transfer, chances to obtain funding increase, allowing to still perform fundamental research in parallel.

Next to the current approaches of SynCellEU to form collaborations with existing companies, we believe that the formation of start-ups is required to create a synthetic cell industry. From our analysis, it followed that it is beneficial for start-ups within new industries to localise into regional clusters. To lay the foundation for a sustainable innovation ecosystem, we therefore believe it is important to first create strong foundations in the form of local synthetic cell hubs. Regionally clustering organisations also allows for an increased level of informal communication, a factor that was found to promote the formation of innovation ecosystems.

Attempts to internalise RRI elements in the local synthetic cell hubs should be actively performed, thereby safeguarding the development of a sustainable innovation ecosystem and increasing the chances of obtaining European funding. When the local synthetic cell hubs have sufficiently devel-

oped themselves, they can be merged into a European innovation ecosystem. On an academic level, communication should already take place across the continent, where the synthetic cell hubs can assist each other in building the regional innovation ecosystems as well.

It also followed from our research that the mere presence of social capital is insufficient to create an innovation ecosystem. Instead, social capital should genuinely be used to increase innovation ecosystem performance. For the development of the local synthetic cell hubs as well as the European innovation ecosystem, it is therefore important to actively seek for relevant collaborations. In this way, SynCellEU can expand through collisions with companies, transfer offices, business incubators, governmental organisations, the public, and other relevant stakeholders, to create a synthetic cell innovation ecosystem in a joint effort.

To conclude, we have multiple suggestions for future research. Firstly, it would be interesting to study how the quantum innovation ecosystem in Delft was formed, and if these insights are valuable in the development of a synthetic cell innovation ecosystem. Secondly, it could be interesting to study American PPPs like Synberc and the Engineering Biology Research Consortium (EBRC), who succeeded in setting up successful companies based on the top-down approach of cell engineering. Thirdly, it would be interesting to study SynCellEU in a broader context. This could be done by taking the universities and research institutes next to TU Delft into account. Finally, future studies could focus more on the drivers to build an innovation ecosystem on an executing level, since we mainly focused on the managerial level.

17

A dual perspective on the future of a synthetic cell

17.1 Expanding through collisions

In this thesis, we described two projects focusing on future expansions of a synthetic cell. In both cases, these expansions are facilitated through collisions. In Part I, we described how the collision between differently sized vesicles can be used to acquire *in vitro* membrane growth. Specifically, we endowed GUVs and LUVs with complementary DNA strands, thereby allowing these two vesicle types to fuse. In a systematic study, we investigated the effects of DNA concentration, lipid composition, vesicle formation method, and other factors on the fusion process. At the same time, we studied how this fusion strategy interacts with a fusion mechanism based on increased membrane tension. Under optimal conditions, we found that up to ~30% of GUVs fused. Although we did not directly observe membrane growth, our findings suggest that fused GUVs exhibited a striking ~1.5-2.8-fold increase in surface area, which would allow for the sustained division of a synthetic cell in the future.

In Part II, we described how collisions between different stakeholders are required in order for a synthetic cell innovation ecosystem to grow. Here, the phrasing "collisions" suggests that collaboration does not come naturally. Indeed, forming collaborations within the innovation ecosystem requires different stakeholders to intensively get to know each other, and to learn from each other's differences in cultures and perspectives. Only in this way, the utilisation of social capital can be increased, which will be the basis of a strong and viable network. By fostering an entrepreneurial culture, promoting the organisation of RRI related activities, and by striving for university-industry collaborations with larger companies, we believe that SynCellEU can facilitate the formation of local synthetic cell hubs across Europe. At a later stage, these local synthetic cell hubs can be combined into a sustainable European innovation ecosystem.

17.2 Personal reflection on the future of a synthetic cell

Throughout this project, I got the opportunity to immerse myself in the fascinating world of synthetic cells for a year and a month. Both projects focused on the expansions of the synthetic cell, and I would like to make use of this section to philosophise on future developments within this field. I attended numerous seminars throughout my project, either organised by the Bionanoscience department at TU Delft, or by departments at other universities or research institutes. Next to this, I read a large number of scientific articles focused on synthetic cell developments. From my personal experience, I noticed that the majority of synthetic cell research is still focused on the development of the individual modules: cell fuelling, DNA processing, and cell division. In fact, many scientific projects, including my own Nanobiology project, only focus on a part of a single module. My project, for example, focused on the acquirement of membrane surface area that is required for a synthetic cell to divide. It should be noted that the approach I adopted, a combination of DNA and tension-mediated vesicle fusion, is only one way to achieve membrane growth. Other fusion mechanisms, for example based on the electrostatic attraction of oppositely charged vesicles [52], are possible as well. Furthermore, membrane growth does not necessarily require vesicle fusion, but can for example also be achieved through the encapsulation of a lipid synthesising minimal genome [34].

Next to membrane growth, synthetic cell division requires the inclusion of other processes that, depending on one's approach, may include the formation of a contractile ring [229], a minimal divisome [230], or the binding of curvature generating membrane proteins [14]. These are just some examples to illustrate that the different synthetic cell modules consist of multiple parts, and that many research groups are working on different approaches to construct these parts. In the end, however, the different parts should be combined with each other into a functioning synthetic cell. This integration of different synthetic cell modules might be an even greater challenge than the realisation of the individual modules. Not only with regard to technical compatibility, but also in terms of collaborations between different research groups. Since many research groups are currently focusing on a single synthetic cell module, the realisation of an integrated synthetic cell might require projects that involve multiple research groups. This will result in additional challenges, where each research group will have their own interests and ideas of what a synthetic cell should look like.

Similar to the realisation of the synthetic cell modules, there are different strategies to build a synthetic cell innovation ecosystem. Here, some strategies might work in a synergistic manner, but others could be conflicting with each other. By first focusing on local synthetic cell hubs across Europe, SynCellEU can probe different strategies to build the foundation of a European innovation ecosystem. It is likely that some action points provided in the advice (Chapter 15) would work out well, but that others require adaptations or a deeper exploration of underlying issues. Furthermore, it is possible that some of the action points work well in specific parts of Europe, but that other places require a different strategy. When the local synthetic cell hubs have been formed, they should be combined into a European innovation ecosystem. Similar to the integration of individual synthetic cell modules, the bundling of synthetic cell hubs might be an even greater challenge than their initial formation. Still, based on the insights obtained in this thesis, I believe that European collaboration is essential to realise synthetic cell applications on the longer term. This will require the integration of different cultures and perspectives, but based on the grand challenges that synthetic cells might tackle in the future, I believe it is worth it to thoroughly invest in this collaboration.

With the integration of different synthetic cell modules, I foresee that more "living-like" systems will be developed in the near future. Once these systems become more advanced, they will become increasingly interesting for industry. It is hard to predict how long it will take before SynCellEU will form its first concrete university-industry collaborations but I foresee that a certain "tipping point" could accelerate the development of an innovation ecosystem. This is also what happened in the case of the quantum ecosystem at TU Delft (Section 10.2), where many start-ups were formed or joined once Microsoft and Intel started to participate in the collaboration. With the action points and intervention provided in the advice (Chapter 15), the formation of the first university-industry collaborations can be promoted. Once the first collaborations have been formed, this could potentially attract additional industry partners and promote the organisation of entrepreneurial initiatives.

The development of more "living-like" systems will also increasingly raise ethical and societal questions. I foresee that the greatest challenge here is to translate theoretical insights into practical actions on the work floor, which requires insights from in-depth case studies similar to the study by Pansera et al. [132] (Subsection 12.2.2). I believe that people should see the added value of RRI related activities and should be intrinsically motivated to engage with RRI. This can be achieved with the action points I provided in Subsection 15.2.2. When I reflect this on myself, I noticed that I had a hard time to actively incorporate RRI in my Nanobiology project, even though I was studying RRI in my Science Communication project. I therefore believe that people should be given the time to both engage with RRI and to implement it in their daily work activities, for example by organising a public engagement event, or by doing a project on improving sustainability in fundamental research.

Despite the great challenges that lie ahead of us, I hope I will contribute to future expansions of a synthetic cell with this thesis, both in terms of technological advancements and fruitful collaborations. Combining these two project certainly has been the most challenging part of my studies, yet it taught me a great deal and I highly enjoyed it.

Tom F. Aarts, March 2022

A

Charge-based experiments

A.1 Materials and methods

Next to performing vesicle binding and fusion experiments based on DNA and tension, charge-mediated experiments were performed in this project. This approach is based on the electrostatic attraction of two vesicle types with opposite charges [79]. For these experiments, GUVs partly consisting of DOPS (PS) lipids were used, giving them a negative charge. Next to this, 30 nm SUVs or 100 nm LUVs partly consisting of DOTAP (TAP) lipids were used, rendering them with positive charge. Both types of vesicles also contained a different lipid dye, allowing for the localisation of the vesicles when illuminated with light with a specific wavelength. We labelled the GUVs with Atto655, and the SUVs with RhoPE. Sizes and stability of charged SUVs and LUVs were determined through DLS measurements as described in Section 3.4.

A.1.1 Charge-mediated GUV-SUV binding

DOPC GUVs containing 20% or 40% DOPS (PS, mol/mol) were prepared as described in Section 3.2, and 30 nm DOPC SUVs containing 20% or 40% DOTAP (TAP, mol/mol) were formed as described in Section 3.3. The following samples were mixed in 20 µL microscopy chambers: 5 µL 20% or 40% PS GUVs with only 15 µL OBS, or with 14 µL OBS and 1 µL 20% or 40% TAP SUVs. After 15 minutes, the samples were imaged using the GUV-0.1%Atto655 and SUV/LUV-RhoPE optical configurations (Table 3.6). For GUV-0.1%Atto655, a LED intensity of 10% was used.

A.1.2 Charge-mediated GUV-SUV fusion

To test fusion efficiency in charge-mediated fusion protocol, we produced 40% PS GUVs and 30 nm 20% TAP SUVs encapsulating 10 mM HPTS. After mixing GUVs and SUVs in quenching OBS and waiting for 10 minutes, we transferred them to imaging chambers for visualisation. The samples were scanned for fused vesicles by looking around in the HPTS channel.

A.1.3 Data acquisition and analysis

All data was acquired using epifluorescence microscopy, except for the second trial of charge-mediated vesicle binding (Subsection A.2.2), where images were taken with confocal microscopy. GUV signal and SUV localisation on the GUV membrane were measured by determining the profile plot intensity of a line traversing a GUV, where a linear fit of the profile plot was subtracted from the data to correct for background signal. The data was subsequently smoothed with a Gaussian filter, and the GUV signal or SUV intensity on the GUV membrane was determined by taking the average peak intensity of the processed profile plot data.

A.2 Results

A.2.1 SUV and LUV characterisation

For both SUVs extruded through a 30 nm pore and LUVs extruded through a 100 nm pore, the fraction of charged lipids did not influence vesicle size (Figure A.1). Similar to what we observed for uncharged vesicles (Subsection 4.2.1), the number PSDs show smaller vesicle diameters than the intensity PSDs. The stability of 40% TAP SUVs and LUVs was assessed, where a dilation in particle size distributions over time was clearly observed for the intensity PSDs (Figure A.1ac), but not for the number PSDs (Figure A.1bd). This corresponds to the fact that larger vesicles can be over-represented in intensity PSDs [91], and could indicate some vesicle aggregation over time. The figure also shows that SUVs can be acquired by directly extruding multilamellar charged vesicles through a 30 nm pore (magenta curves), instead of first pushing them through a 100 nm pore as done by Deshpande et al. [30]. Next to this, the figure shows that the diameter of 100 nm LUVs decreased after 9 extrusion steps through a 30 nm pore, but that the vesicle diameter became smaller after 21 extrusion steps. This is in contrast to what we found for uncharged vesicles, where we found comparable vesicle diameters after 4 and 22 extrusion steps (Figure 4.2ab).

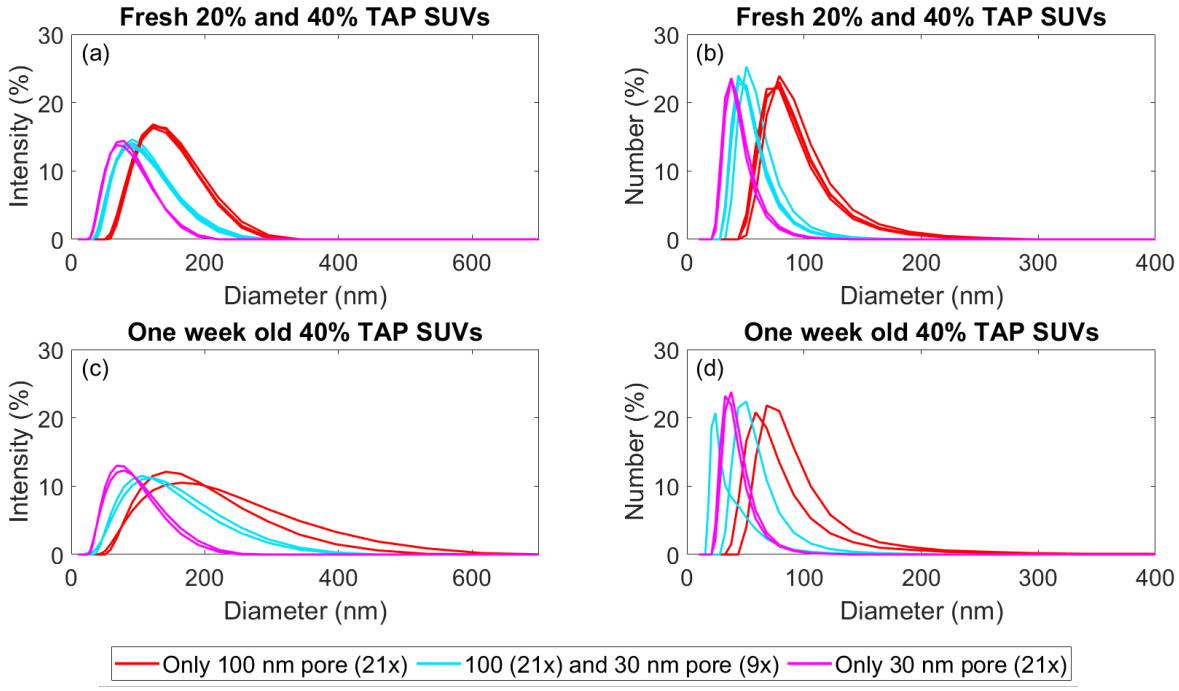


Figure A.1 DLS measurements to verify sizes of 20% and 40% TAP 30 nm SUVs and 20% and 40% TAP 100 nm LUVs. Sub-figures (a) and (b) show data from both 20% and 40% TAP vesicles, indicating that the magnitude of charge had no effect on vesicle size. The results are displayed in intensity (a & c) and number PSDs (b & d), and are done on fresh (a & b) and one week old vesicles (c & d). Each graph represents a single measurement which is an average over an automatic number of runs. Vesicles were either extruded 21 times through the 100 nm pore, first 21 times through the 100 nm pore and then 9 times through the 30 nm pore, or directly 21 times through the 30 nm pore.

A.2.2 Charge-mediated GUV-SUV binding

First trial (epifluorescence microscopy)

The first trial for charge-mediated GUV-SUV binding was imaged with fluorescence microscopy. Both control samples containing no SUVs showed a signal in the SUV channel (Figures A.2a and A.2c). This signal was probably due to fluorescence crosstalk of Atto655 into the SUV channel. Crosstalk of Atto655 complicated data analysis, since signal in the SUV channel could be caused by both GUV ans SUV lipid dyes in the presence of SUVs. To still interpret the data in a qualitative way, we made

composite images for the different conditions of the experiment (Figure A.2, third column). Here, we can appreciate that both control samples (a and c) and the show a homogeneous composite signal. This indicates that the signal in the GUV and SUV channels are similar, suggesting that the signal in the SUV channel was indeed caused by crosstalk. In the sample with 20% charged GUVs and SUVs (b), a homogeneous composite signal is visible as well. This suggests that the electrostatic interactions of these two vesicle types were not strong enough to let them bind to each other, or that SUVs were homogeneously bound to GUVs. In the sample with 40% charged GUVs and 20% charged SUVs (d), however, a clear heterogeneous composite signal is visible, where most GUVs show a higher SUV signal, but some (parts of) GUVs also show a higher GUV signal. The increased SUV signal on the majority of GUV membranes indicates localised binding of SUVs to GUVs.

We quantified the SUV signal on the GUV membrane for the different conditions tested. For 20% PS GUVs, we found no significant increase in SUV signal for 20% TAP SUVs compared to no SUVs (Figure A.3a, one-way ANOVA, $p = 1.00$). For 40% PS GUVs, on the other hand, we found a significantly higher SUV signal on the GUV membrane for 20% TAP SUVs compared to using no SUVs (Figure A.3b, one-way ANOVA, $p = 0.011$). For 40% PS GUVs with or without SUVs, we found no significant difference in GUV signal (one-way ANOVA, $p = 0.85$), showing that the increase in SUV signal was not caused by a change in GUV signal. For 20% PS GUVs, we did find a significant decrease in GUV signal for the GUVs mixed with SUVs (one-way ANOVA, $p = 0.020$), indicating sample heterogeneity in GUV signal.

The findings are in line with the increased SUV signal we observed on the membrane of 40% PS GUVs mixed with 20% TAP SUVs (Figure A.2d). The results also suggest that the homogeneous signal observed for 20% PS GUVs and 20% TAP SUVs (Figure A.2b) indicates that SUVs did not bind sufficiently to the GUV membrane for this condition to observe GUV-SUV binding.

Second trial (confocal microscopy)

We performed a second trial for charge-mediated GUV-SUV binding. Next to the conditions tested in the first trial, we included 40% TAP SUVs in this experiment. Another difference with the first trial is that this experiment was imaged with confocal microscopy instead of epifluorescence microscopy. We observed a high SUV background signal for this experiment (Figure A.4), which was possibly caused by erroneous filter settings. The high background signal complicated both qualitative and quantitative interpretation of this experiment. Still, we observed an increased SUV signal on the GUV membrane for 40% PS GUVs and 20% TAP SUVs (Figure A.4e), which is in line with the results of the first trial (Figure A.3d). For the other conditions, including the samples containing SUVs with a larger fraction of charged lipids (40% TAP), we observed no clear SUV localisation on the GUV membrane. We hypothesise that this was caused by a more rapid neutralisation of the GUV membrane upon binding with 40% TAP SUVs. Lira et al. indeed pointed out that charge-mediated fusion is expected to continue up to charge neutralisation [52]. We thus expect that some GUV-SUV binding occurred for 40% TAP SUVs, but that the degree of binding was insufficient for us to observe it with confocal microscopy.

In contrast to the first trial, we found no significant differences in SUV signal on the GUV membrane when adding no SUVs, 20% TAP SUVs, or 40% TAP SUVs to 20% or 40% PS GUVs (Figure A.5). Still, we found the highest levels of GUV-SUV binding for 40% PS GUVs and 20% TAP SUVs, the condition that showed significant GUV-SUV binding in the first trial (Figure A.3). Possibly, we observed no significant binding in the second trial due to the low number of GUVs analysed ($n = 10$ for 40% PS GUVs), and/or due to the difficulty to obtain quantitative results with the high SUV background signal.

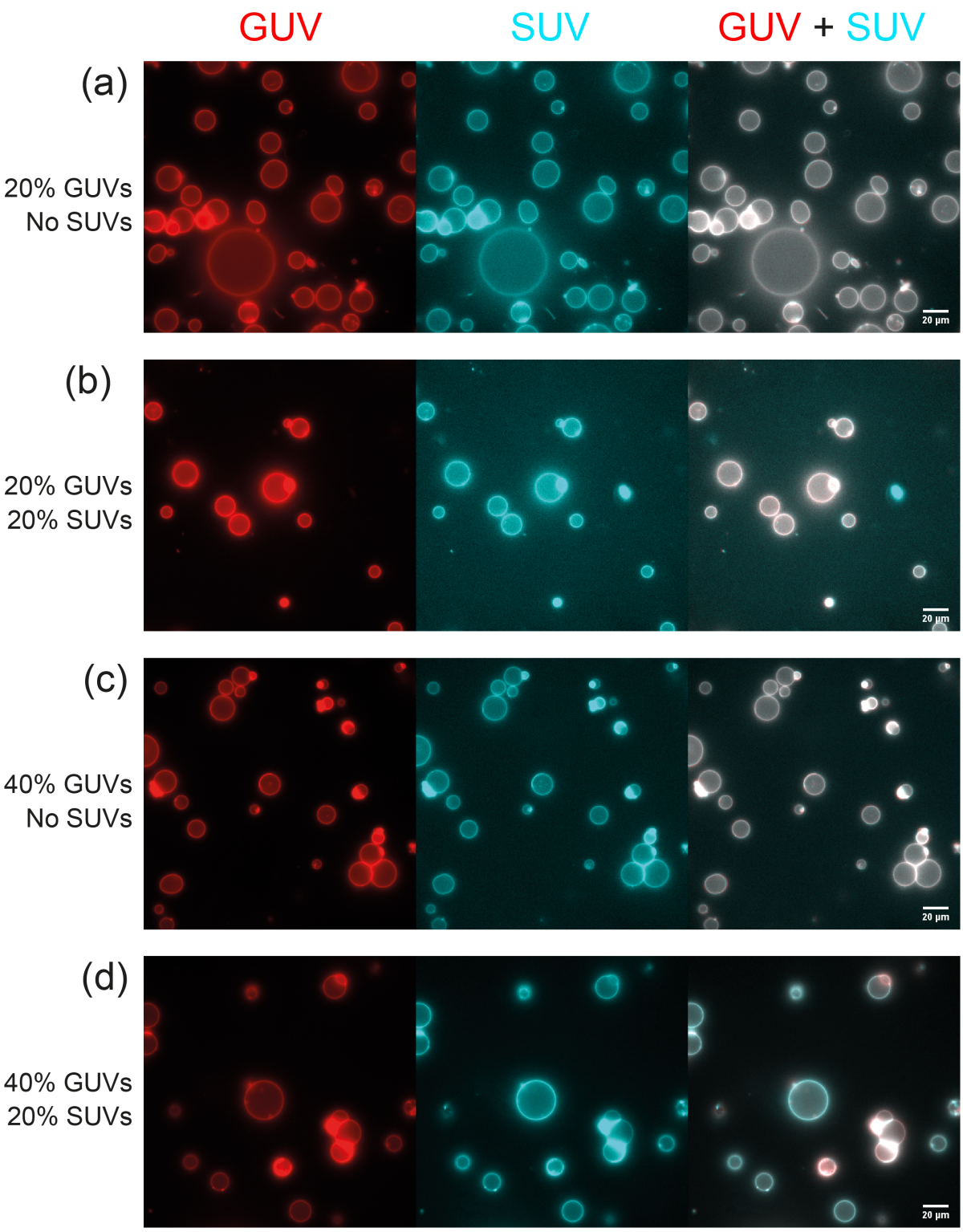


Figure A.2 Qualitative results of the first trial of charge-mediated GUV-SUV binding. We included 20% PS GUVs without SUVs (a), 20% PS GUVs with 20% TAP SUVs (b), 40% PS GUVs without SUVs (c), and 40% PS GUVs with 20% TAP SUVs (d). The first column shows the signal of the GUV channel, the second column of the SUV channel, and the third column shows a composite of the two individual channels. The controls without SUVs in (a) and (c) show a signal in the SUV channel, probably due to crosstalk with the Atto655 (GUV) dye. Both control samples and sample (b) form a composite image with a homogeneous signal. Sample (d) shows a heterogeneous signal in the composite images, indicating localisation of SUVs on the GUV membrane. Images were obtained with epifluorescence microscopy. Scale bars indicate 20 µm.

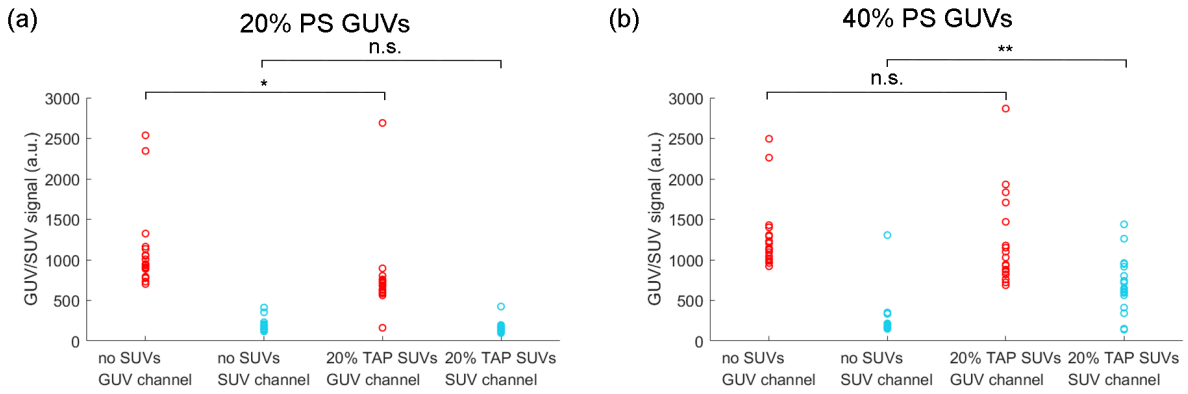


Figure A.3 Semi-quantitative results of the first trial of charge-mediated GUV-SUV binding. We included 20% PS GUVs (a) and 40% PS GUVS (b) with no SUVs or 30 nm 20% TAP SUVs. The GUV channel shows the background-subtracted signal of the GUV membrane, and the SUV channel shows the background-subtracted SUV signal on the GUV membrane. We found non-significant (n.s.) and significant relationships ($p \leq 0.05$ for *, $p \leq 0.01$ for **). The sample size for each condition was n = 20.

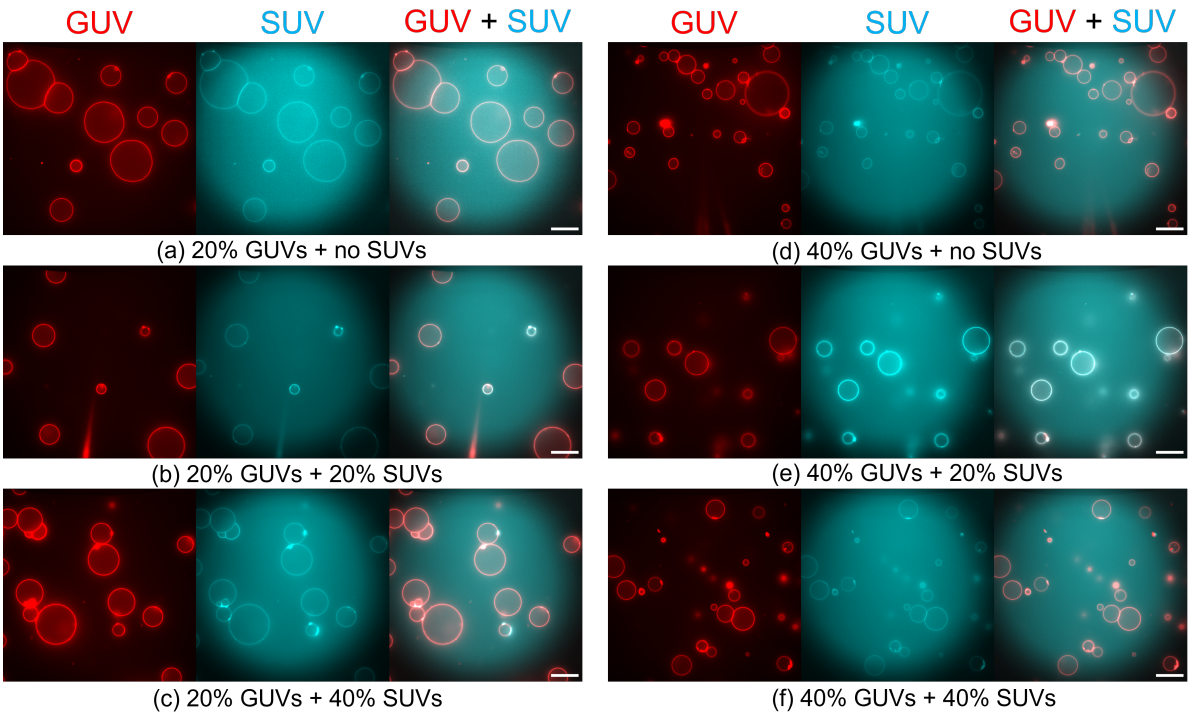


Figure A.4 Qualitative results of the second trial of charge-mediated GUV-SUV binding. Images are shown in GUV, SUV and composite (GUV + SUV) channels. GUV channels of Sub-figures (b-f) have equal brightness and contrast, as well as the SUV channels of these images. Brightness and contrast of Sub-figure (a) was adapted since this image showed a lower GUV and SUV signal, possibly caused by different imaging settings. GUV-SUV binding was only observed for 40% PS GUVs and 20% TAP SUVs. Images were obtained with confocal microscopy and showed a high SUV background signal, possibly caused by erroneous filter settings. Scale bars indicate 30 µm.

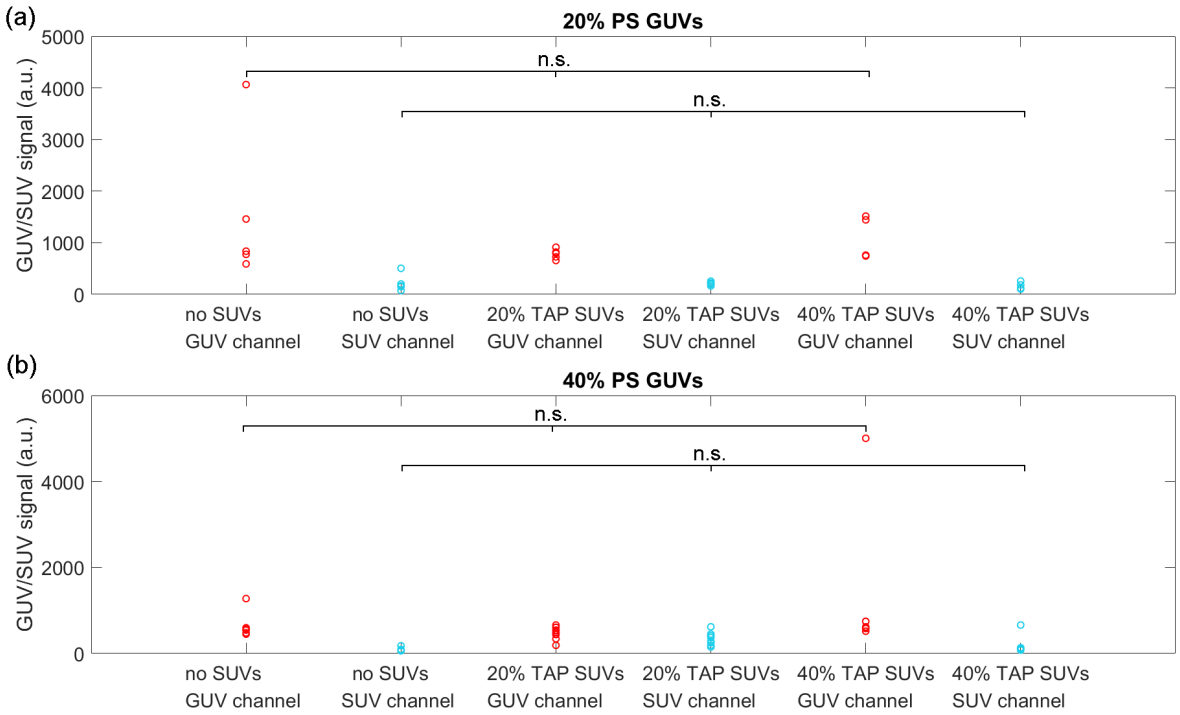


Figure A.5 Semi-quantitative results of the second trial of charge-mediated GUV-SUV binding. Charge-mediated binding of 20% PS GUVs (a) and 40% PS GUVs (b) with no SUVs, 20% TAP SUVs, or 40% TAP SUVs. GUV channel shows the background-subtracted signal of the GUV membrane, and SUV channel shows the background-subtracted SUV signal on the GUV membrane. We only found non-significant (n.s.) relationships, possibly caused by the small sample sizes (n = 5 for the 20% PS GUV samples and n = 10 for the 40% PS GUV samples) or the high SUV background signal.

A.2.3 Charge-mediated GUV-SUV fusion

In Section A.2.2, we found that SUVs localise on GUV membranes for 40% PS GUVs and 20% TAP SUVs. To determine if these vesicles undergo membrane fusion, we performed the HPTS-DPX content mixing assay (Section 4.6) on charged vesicles. Here, we found some instances of vesicle fusion (Figure A.6a-d), but this was not a regular phenomenon (~10 GUVs per sample were fused). In general, the fused GUVs were relatively small, corresponding to earlier findings showing that smaller vesicles are more fusogenic [42, 43]. We also observed many GUVs sticking to each other or to the surface (Figure A.6ef). Earlier work did show successful charge-mediated GUV-SUV/LUV fusion [e.g. 52, 84]. In our case, the low fusion efficiency and vesicle stickiness was possibly caused by our vesicle formation methods, lipid compositions and/or imperfect microscopy chamber surface passivation. We concluded that our protocol for charge-mediated fusion was not useful to obtain GUV membrane growth.

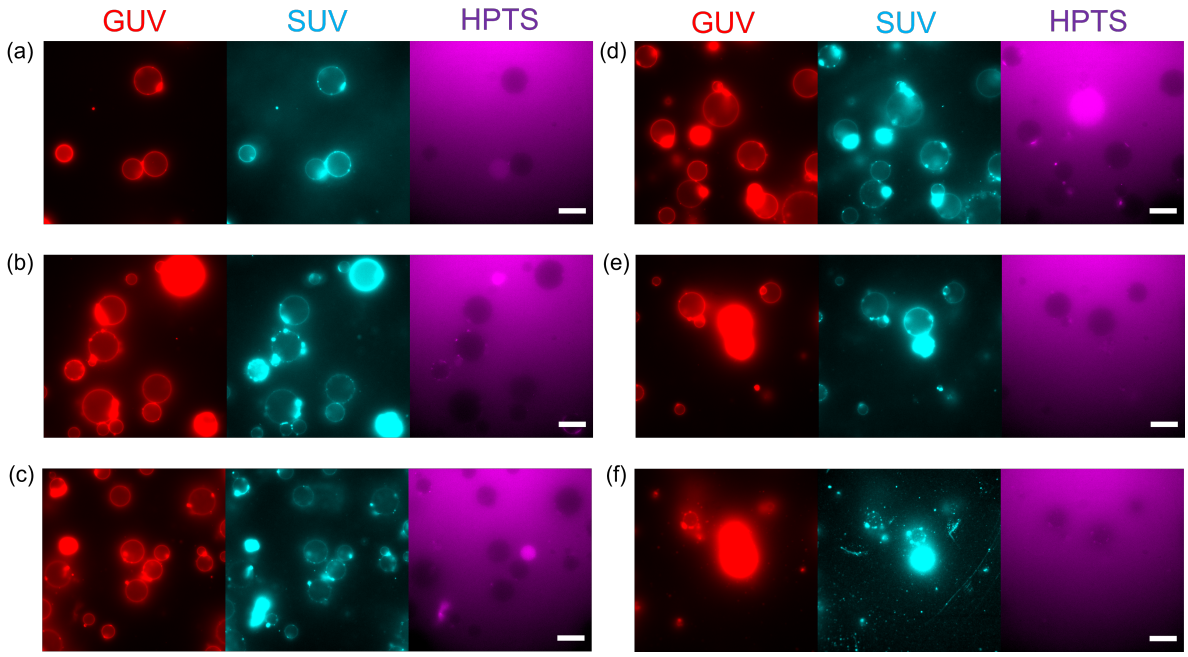


Figure A.6 Charge-mediated GUV-SUV binding. 40% PS GUVs were mixed with 20% TAP SUVs. Fused vesicles were observed (a-d), but fusion was not a regular phenomenon (~10 per sample). Scale bars indicate 20 µm. GUVs were often sticking to each other, and SUVs were often locally clustered. Sub-figures (e) and (f) are taken at the same FOV but at a different focus, and show vesicles sticking to the surface.

B

Systematic check of the working fusion protocol

We found that fusion efficiency depended dramatically on the order of mixing vesicles (Subsection 4.7.6). In this appendix, we will discuss the systematic check we performed to investigate why fusion was promoted by the working fusion protocol. This systematic check was performed before we came up with the current hypothesis for the efficacy of the working fusion protocol: we propose that the difference in fusion results is caused by dilution of the sample prior to GUV-LUV binding leading to DNA desorption for the ineffective fusion protocol (Subsection 5.8.3).

B.1 Materials and methods

B.1.1 Osmotic shock

The first factor assessed that could explain the enhanced fusion rate was the osmotic shock that GUVs experience when they are incubated with LUVs alone (Subsection 3.11.3). This was assessed in parallel with the experiment where the effect of osmotic conditions on GUV-LUV binding was investigated 3.8.3). By incubating the GUVs and LUVs in superhypotonic OBS (Table 3.11), an osmotic shock similar to the working fusion protocol was induced. By looking around in the HPTS channel, it was investigated if vesicles had fused.

B.1.2 Quenching OBS components

It was also tested if components that were present in the quenching OBS, but not in the GUV swelling buffer and SUV/LUV buffer, could explain the enhanced fusion rate of the working fusion protocol. The components we tested are the quencher DPX and glucose. This was done by incubating 0.5% Atto488 GUVs with 2.5 µM chol-DNA2-x (overnight without mixing) and 200 nm Atto655 LUVs with 2.5 µM x-DNA1-chol (overnight without mixing). The next day, 10 µL GUVs and 10 µL LUVs were either incubated with 20 µL GUV swelling buffer (containing no glucose and DPX) or with 20 µL quenching OBS (containing glucose and DPX). After 70 minutes, 20 µL of the buffers were added to the samples in such a way that they had the same final composition. Then, the effect of adding quenching OBS after 70 minutes could be tested by transferring the samples to 200 µL microscopy chambers and by looking around in the GUV channel.

B.1.3 Vesicle density

When GUVs and LUVs are directly put in quenching OBS, the vesicle density is lower compared to the working fusion protocol. To assess if this lower vesicle density could explain the difference in fusion rate between the two fusion protocols, longer waiting steps for the vesicles directly put in quenching OBS were performed. Here, it was assumed that a lower vesicle density could slow down the fusion process, but that similar fusion rates should be achieved with longer waiting steps. The experiments where the effect of these longer waiting steps was assessed are displayed in Table B.1.

Experiment	Waiting step
Vesicle mixing order and KCl concentration (Subsection 3.11.7)	2-3.5 hr ([KCl] = 10 mM)
Systematic check working fusion protocol: Osmotic shock (Subsection B.1.1)	2 hours

Table B.1 Experiments where the effect of longer waiting steps on fusion rate was tested.

B.2 Results

B.2.1 Osmotic shock

One possible explanation for the efficacy of the working fusion protocol is the osmotic shock GUVs experience during GUV-LUV incubation (Subsection 4.7.2). By directly adding GUVs and LUVs to quenching OBS, the osmotic difference between the GUV exterior and interior is lower than in the case of the working fusion protocol, possibly impeding fusion. To test this, we lowered the osmolarity of quenching OBS. By directly adding GUVs and LUVs to this OBS with a decreased omsolarity, osmotic conditions similar to the GUV-LUV incubation step of the working fusion protocol were recreated. Despite the fact that LUVs were bound to GUVs, we found no single clearly fused vesicle (Figure B.1). The enhanced fusion rate of the working fusion protocol can thus not be explained by the osmotic shock present during GUV-LUV incubation.

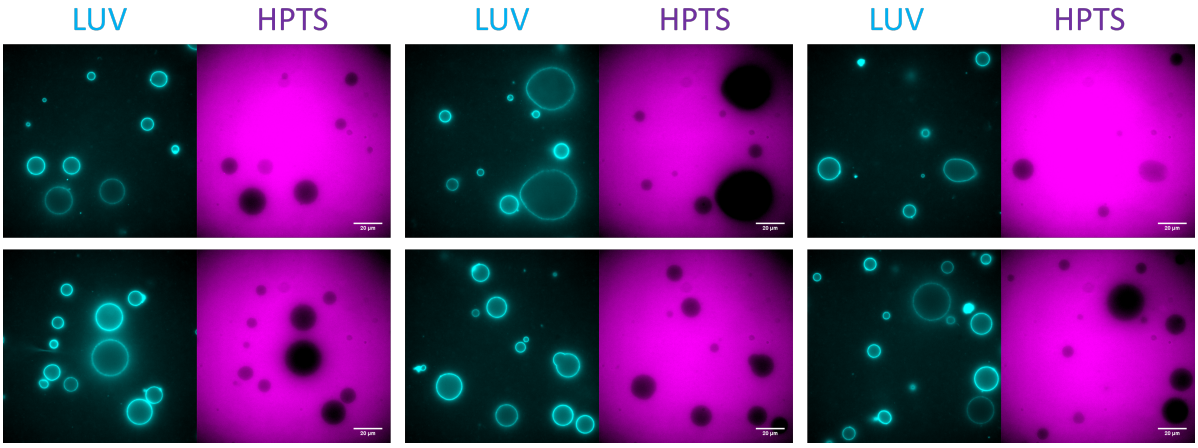


Figure B.1 Results of osmotic shock control to investigate the efficacy of the working fusion protocol. Osmotic conditions similar to the GUV-LUV incubation step of the working fusion protocol were recreated, but no single clearly fused vesicle was observed. Scale bars indicate 20 µm.

B.2.2 Quenching OBS components

Another possibility is that components in quenching OBS prevent the occurrence or detection of fusion. Consequently, we would not be able to observe fusion by directly adding GUVs and LUVs to quenching OBS. To test this possibility, we added GUVs and LUVs to swelling buffer instead of quenching OBS after DNA incorporation, thereby excluding the quenching OBS components DPX and glucose during potential fusion. These components were then later added to the samples to obtain a sample composition comparable to the working fusion protocol (Subsection B.1.2). Similar to the osmotic shock assessment, LUVs clearly localised on the GUV membrane, but we found no single fused vesicle (Figure B.2). This indicates that the quenching OBS components we tested are not responsible for the low fusion rate observed when directly adding vesicles to quenching OBS. Next to this, we excluded another factor that could have explained the effectiveness of the working fusion protocol. Since GUVs contain the monosaccharide glucose and OBS contains the disaccharide sucrose, GUVs are confined to a small region at the bottom of the chamber. In contrast, GUVs diffuse through three-dimensional space during the GUV-LUV incubation step of the working fusion protocol, since glucose is not yet present at this step. The higher dimensionality of the GUV-LUV incubation step could have explained

the effectiveness of the working fusion protocol, since this would possibly allow for more GUV-LUV interactions. By excluding glucose from quenching OBS in this experiment, vesicles could still freely diffuse after addition to quenching OBS, but as shown, no fused vesicles were observed.

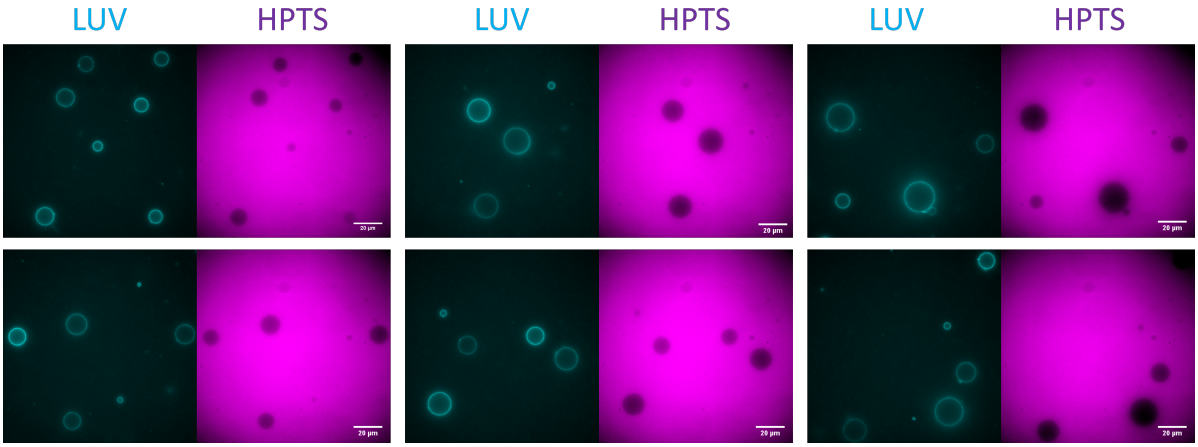


Figure B.2 Results of quenching components control to investigate the efficacy of the working fusion protocol. LUVs clearly localise on the GUV membrane, but no single fused vesicle was observed. Scale bars indicate 20 µm.

B.2.3 Vesicle density

The final factor we tested in the systematic check of the working fusion protocol is vesicle density. In our fusion protocol, the vesicle density of GUVs and LUVs is halved after adding them to quenching OBS (Section 3.10). Possibly, this reduced vesicle density decreased the fusion rate due to a lower number of GUV-LUV interactions. We hypothesised that a decreased vesicle density could reduce the fusion rate, but that by waiting sufficiently long, similar fusion rates as the working fusion protocol should be detected. In the working fusion protocol, we incubated GUVs and LUVs for 60-80 minutes before adding them to quenching OBS. The results in Subsection B.2.2 were obtained after 2 hours, but no single fused vesicle was observed here. Next to this, the vesicles of the ineffective fusion protocol in Subsection 4.7.6 were observed after 2-3.5 hours, but these vesicles neither showed fusion.

C

Additional fusion detection methods

C.1 Materials and methods

Next to the HPTS-DPX content mixing assay we described in the main text (Section 4.6), we tested several other methods to detect fusion. Details of the (fluorescent) compounds described in this appendix are displayed in Tables 3.1 and 3.2. Next to this, the complete lipid compositions of vesicles are displayed in Tables 3.4 and 3.5.

C.1.1 Calcein-cobalt content mixing assay

Fusion has been detected with a calcein-cobalet content mixing assay in previous work [54, 231, 232]. This method is based on the ability of cobalt ions to quench the fluorescent molecule calcein, which is reversed by the ability of EDTA to chelate cobalt ions. By encapsulating calcein and cobalt ions in one vesicle type, and EDTA in the other vesicle type, fusion can be detected from a fluorescent calcein signal upon successful vesicle fusion (Figure C.1).

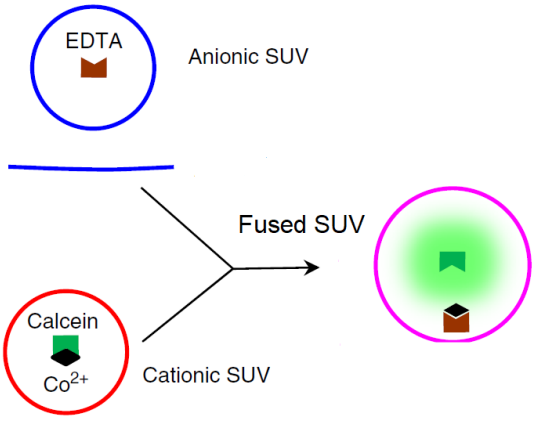


Figure C.1 The calcein-cobalt content mixing assay to detect vesicle fusion. Fluorescent calcein is added to one vesicle type, that is quenched by cobalt ions. Another vesicle type contains EDTA, that chelates cobalt upon successful vesicle fusion, causing calcein to fluoresce. Figure adapted from Ishmukhametov et al. [54].

We investigated the potential of this method for our experiments by encapsulating calcein and cobalt in 30 nm SUVs and EDTA in GUVs. For this, 10 mM EDTA was added to GUV swelling buffer, and 1 mM $CoCl_2$ and 1 mM calcein was added to SUV/LUV buffer. To quench calcein in the outer vesicle solution, 1 mM $CoCl_2$ was added to OBS. To test this method for charge-mediated fusion, vesicles were prepared and observed in these adjusted buffers, and the method was tested for charge-mediated fusion. For this, 40% PS GUVs and 30 nm 20% TAP SUVs were used. To wash off external EDTA from the GUVs, they were diluted 10 times in a 1.5 mL Eppendorf Tube ® with normal OBS. After 15 minutes, the GUVs were pipetted from the bottom of the tube and diluted again 10 times with normal OBS. This dilution step was repeated once. Since there was leakage during SUV formation in this case (see Subsection 3.3.2), the 20% TAP SUVs were extruded only 11 times through the 100 nm pore and 9 times through the 30 nm pore, and the 40% SUVs were extruded 21 times through the 100 nm pore

and 9 times through the 30 nm pore. To test for fusion, 20 µL GUVs and 1 or 4 µL SUVs were added to 60 µL OBS with cobalt ions in a 200 µL microscopy chamber.

For the DNA-based calcein-cobalt trial, 0.1% Atto655 GUVs were prepared with GUV swelling buffer containing 10 mM EDTA, and 200 nm RhoPE LUVs were prepared with SUV/LUV buffer containing 1 mM $CoCl_2$ and 1 mM calcein. To wash off external EDTA from the GUVs, they were diluted 10 times with normal OBS in a 1.5 mL Eppendorf Tube ®. After a sedimentation step of 4 hours, 39 µL GUVs were retrieved from the bottom of the tube, to which 1 µL chol-DNA2-x was added to obtain a DNA concentration of 2.5 µM. Similarly, the LUVs were incubated with x-DNA1-chol to also obtain a DNA concentration of 2.5 µM. Both GUVs and LUVs were incubated with DNA overnight. The next day, 10 µL GUVs and 10 µL LUVs were added to a 1.5 mL Eppendorf Tube ® containing 20 µL OBS with 1 mM $CoCl_2$. After an incubation step of 75 minutes, the sample was pipetted into a microscopy chamber of 200 µL to check for fusion.

For all calcein-cobalt content mixing experiments, the GUV-0.1%Atto655, SUV/LUV-RhoPE and calcein optical configurations were used to detect vesicles and fusion. For GUV-0.1%Atto655, a LED intensity of 5% was used. Fused vesicles were tracked down by looking around in the calcein channel.

C.1.2 R18 self-quenching assay

Alternative to content mixing assays, fusion can also be detected by lipid mixing assays. The self-quenching dye R18 was used to test another fusion assay. Based on work by Exterkate et al. [233], we hypothesised that by incorporating R18 in GUVs at a self-quenching concentration, the R18 fluorescence would increase upon fusion with smaller vesicles due to R18 dilution. A stock solution of 10 mg/mL R18 was prepared by diluting 10 mg R18 in a solution of 0.5 mL chloroform and 0.5 mL methanol. Two approaches to incorporate R18 in the GUV membrane were performed. In the first approach, R18 was incorporated during vesicle formation by preparing R18 GUVs. In the second approach, unlabelled GUVs were prepared, to which 2.5, 5 or 7.5% R18 was added overnight. Next to this, 2.5 µM chol-DNA2-x was incubated overnight will all GUV types. The next day, 5 µL of each GUV type was added to 15 µL OBS in a 20 µL microscopy chamber. By using the GUV-R18 optical configuration, R18 fluorescence on the GUV membrane could be determined.

To assess if R18 could be used to detect GUV-LUV fusion, unlabelled 200 LUVs were prepared. Here, no lipid dye was used, since the excitation spectrum of the most frequently used lipid dye for LUVs (Atto655) covers the excitation peak of R18. Next to this, it was not found necessary to include a lipid dye here, since earlier experiments in general showed no issues for GUV-LUV binding. Part of the LUVs were incubated overnight with 2.5 µL x-DNA1-chol. The next day, 10 µL R18 GUVs (with DNA) were incubated with 10 µL LUVs (with or without DNA) for ~75 minutes. Then, the vesicles were added to 200 µL microscopy chambers containing 20 µL OBS. Similar to the R18 incorporation assessment, GUV-R18 fluorescence was determined using the R18 optical configuration. To prevent evaporation, the microscopy chambers were closed with lids.

C.1.3 Streptavidin-biotin fusion assay

With the goal to be able to detect live fusion, a fusion assay based on the strong binding affinity of streptavidin and biotin was tested [234]. In this fusion assay, 0.5 µM fluorescent streptavidin was encapsulated in unlabelled GUVs (Figure C.2a). Next to this, 20 mol% Biotinyl PE was incorporated in 200 nm LUVs. We hypothesised that through DNA-mediated GUV-LUV fusion, biotin would be incorporated into the GUV membrane, resulting in the localisation of streptavidin on the GUV membrane (Figure C.2b). Here, 1 µM chol-DNA2-x was added to GUVs, and 1 µM x-DNA1-chol was added to LUVs. Both vesicle types were incubated overnight with DNA. In reality, streptavidin was also present in the outer vesicle solution (Figure C.2c). This external streptavidin was able to bind to LUVs that were bound to GUVs, but that did not necessarily undergo full fusion. To be able to detect vesicle fusion, external streptavidin had to be degraded by adding Proteinase K (Figure C.2d). This was done by adding 0.4 µL Proteinase K to 25 µL GUVs in 49.6 µL OBS, resulting in a Proteinase K concentration of 1 mg/mL.

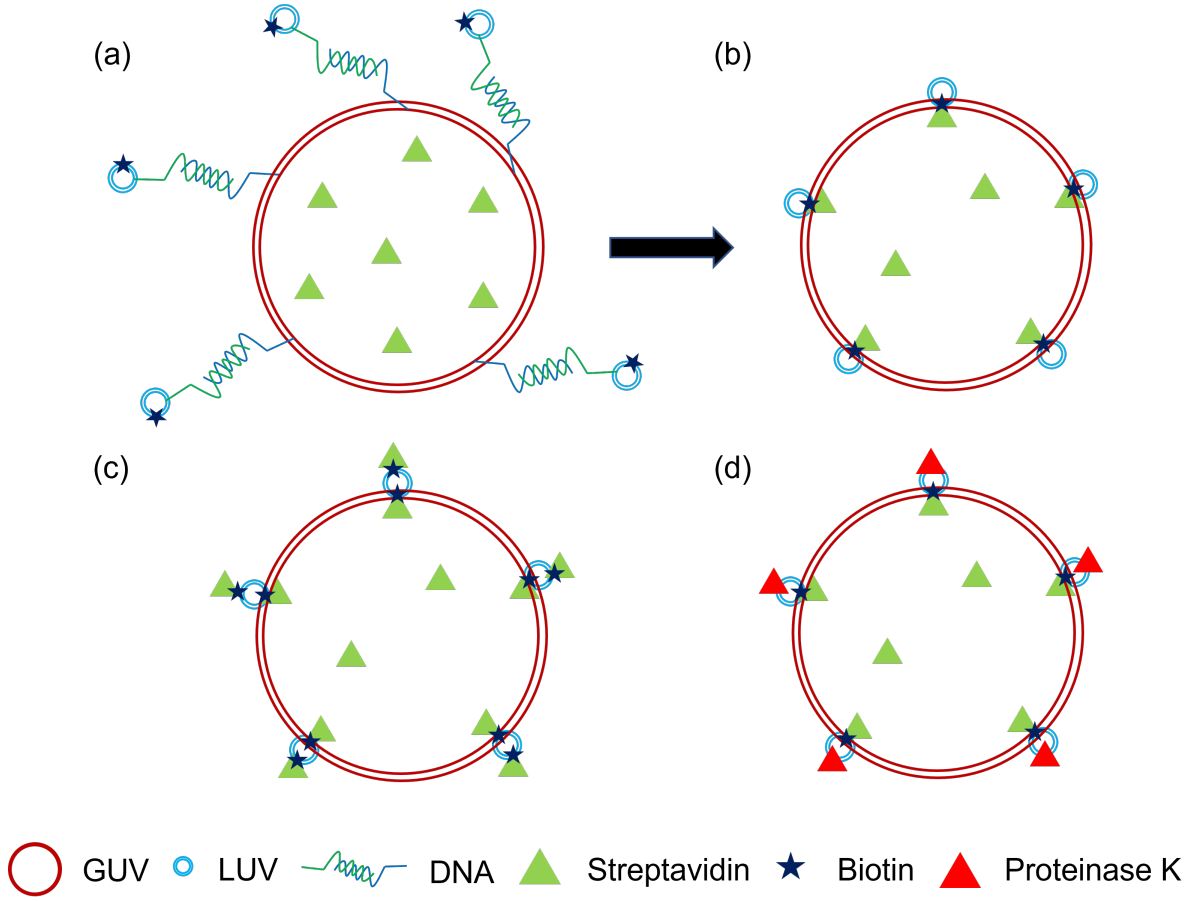


Figure C.2 The proposal for a streptavidin-biotin assay to detect vesicle fusion. GUVs are encapsulated with fluorescent streptavidin, and biotin is incorporated in LUV membranes (a). Through DNA-mediated fusion, biotin is incorporated into the GUV membrane, causing streptavidin to localise on the GUV membrane (b). In reality, streptavidin is also present in the outer vesicle solution, that can bind to LUVs that are bound but not necessarily fused with GUVs (c). Proteinase K is added to the outer vesicle solution to degrade external streptavidin (d).

To test the influence of incubation time and temperature on Proteinase K activity, the GUVs were either incubated for 30 minutes at room temperature, or for one hour at 37℃. Then, the level of streptavidin encapsulation was assessed by transferring the samples to 200 µL microscopy chambers and by using the Streptavidin-Alexa488 optical configuration. To assess if the streptavidin-biotin assay is able to detect fusion, 25 µL LUVs were added to each sample without mixing. After approximately one hour, the level of LUV binding and changes in streptavidin signal were observed using the SUV/LUV-Atto655 and Streptavidin-Alexa488 optical configurations.

Since a low level of LUV binding was observed for the sample that was incubated at 37℃ (Figure C.8b), potentially due to a lack of mixing, the level of DNA incorporation at this temperature was tested. For this, 0.01% Atto655 GUVs were prepared. The ability of DNA to incorporate at 37℃ was tested at multiple steps of the protocol. This was done by adding 1 µM chol-DNA2-x and 1 µM x-DNA1-Atto488 to GUVs in 1.5 mL Eppendorf Tubes ® at different time points (Table C.1): one hour prior to (sample 1), two minutes before (sample 2), or 30 minutes after (sample 3) incubating the vesicles for one hour at 37℃. Next to this, the same ssDNAs were incubated with GUVs at room temperature (sample 4). The incubation times for the different samples can also be found in Table C.1. Since DNA was added at different steps in the protocol, the variation in DNA incubation times is quite large. However, it was shown that the level of DNA incorporation after 5 minutes and an overnight incubation is comparable (Subsection 4.3.3). We therefore assumed that the differences in DNA incubation time had no influence on the level of DNA incorporation. The degree of DNA incorporation could be measured by transferring part of the vesicles to 200 µL microscopy chambers containing OBS, and by making images using the GUV-0.01%Atto655 and DNA-Atto488 optical configurations.

Sample	DNA incubation temperature	DNA incubation time (min.)	DNA incubation time mixed samples (min.)
1	37°C	150	230
2	37°C	100	165
3	37°C	20	40 & 70
4	Room temperature	140	165 & 180

Table C.1 Samples used to determine the level of DNA incorporation at 37°C during multiple steps of the protocol. Samples were mixed by pipetting the solutions up and down three times. For mixed samples with multiple DNA incubation times, the samples were only mixed before the first time point.

Since there was quite some variation in Atto488 signal for the different samples (Figure C.9), the samples were observed again after a mixing step. DNA in free vesicle solution can potentially bind to the glass surface of a microscopy chamber for longer time points (Subsection 4.3.3). The mixing step was therefore performed on the remaining GUVs in the Eppendorf Tubes ®. This was done by pipetting the solutions up and down three times. Then, the freshly mixed vesicles were transferred to new 200 µL microscopy chambers containing OBS. The DNA incubation times for the mixed samples can be found in Table C.1. The samples that have multiple incubation times after mixing were only mixed before the first time point.

C.1.4 His-nickel fusion assay

For the streptavidin-biotin fusion assay, we still observed external streptavidin signal after adding Proteinase K (Figure C.7). We hypothesise that this was caused by Proteinase K only degrading the streptavidin protein but not its fluorescent tag. We therefore suggest an alternative fusion assay that is also based on the the strong binding affinity of two compounds: His-tagged proteins and nickel ions [235]. We specifically suggest to encapsulate His-GFP in GUVs and to incorporate nickelated lipids in LUVs. Similar to the streptavidin-biotin fusion assay, we hypothesise that His-GFP will localise on the GUV membrane upon successful GUV-LUV fusion. The advantage of this assay is that the His-GFP itself is fluorescent, in contrast the non-fluorescent streptavidin protein in the streptavidin-biotin fusion assay. We hypothesise that addition of Proteinase K to the outer vesicle solution will fully degrade external His-GFP, thereby eliminating external His-GFP signal.

The ability of Proteinase K to degrade His-GFP was measured with a bulk fluorescence experiment. Here, experimental conditions similar to fusion experiments were recreated by adding 0.5 µM His-GFP to a solution of OBS buffer, GUV swelling buffer and SUV/LUV buffer in a 2:1:1 volume ratio. His-GFP was incubated in the presence or absence of 0.1 mg/mL Proteinase K for one hour at room temperature or at 37℃. Then, His-GFP fluorescence was measured with a plate reader (Infinite M200 Pro, Tecan Group Ltd.) in a dark 384 well plate (MaxiSorp™ 384 well plates, Nunc™). The samples were illuminated with both the first (395 nm) and second (475 nm) excitation peaks of GFP, and fluorescence was measured at the emission peak of GFP (508 nm) using gains of 100, 110 (optimal), and 115.

C.2 Results

C.2.1 Calcein-cobalt content mixing assay

The ability to detect fusion with the calcein-cobalt content mixing assay was tested both for charge and DNA-mediated fusion. We aimed to wash off external calcein by performing multiple washing steps (Subsection C.1.1), resulting in a relatively low GUV density (Figure C.3). Despite the washing steps and the external addition of cobalt ions, we still observed some external calcein signal. Throughout the sample, we observed a single GUV with an internal calcein singal, suggesting that this GUV underwent vesicle fusion (Figure C.3b). At the microscopy chamber surface, we observed aberrant structures involving tubular structures and aggregation of SUVs (Figure C.3cd).

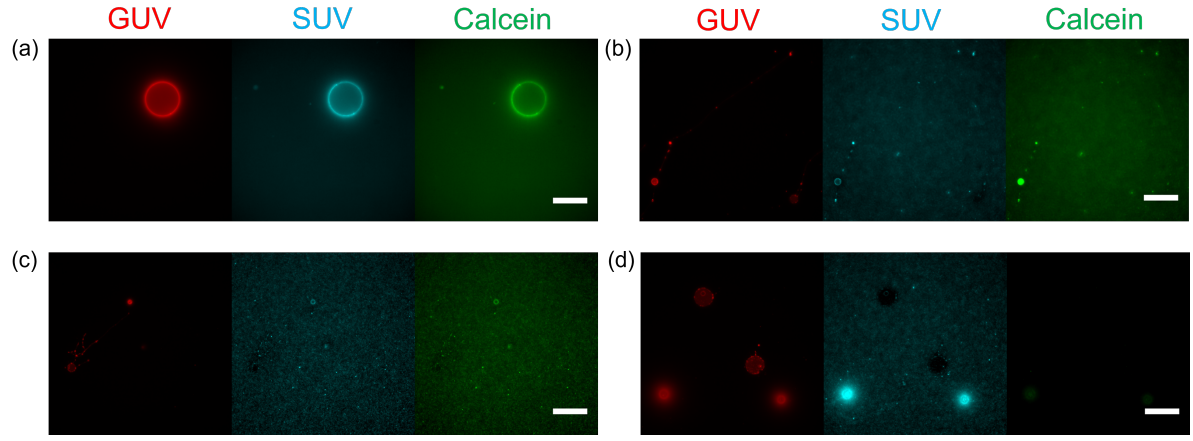


Figure C.3 Detection of charge-mediated GUV-SUV fusion with the calcein-cobalt content mixing assay. 40% PS GUVs were mixed with 30 nm 20% TAP SUVs. The GUV density was relatively low, and one fused vesicle was observed (b). Aberrant structures were observed at the surface (c and d). Scale bars indicate 40 µm.

For the experiment where we tested the calcein-cobalt content mixing assay for DNA-mediated fusion, we performed a single washing step to wash off external EDTA (Subsection C.1.1). We hypothesise that this led to a higher vesicle density compared to the charge-based experiment, but also in a higher calcein background signal (Figure C.4), caused by residual EDTA dequenching external calcein. We observed a relatively low level of GUV-LUV binding for this experiment, which can be judged from the speckled LUV signal on the GUV membrane [82], and the high LUV background signal. We hypothesise that this is caused by external cobalt ions binding to DNA, thereby preventing GUV-LUV binding. No single fused GUV was found using the calcein-cobalt content mixing assay to detect DNA-mediated fusion.

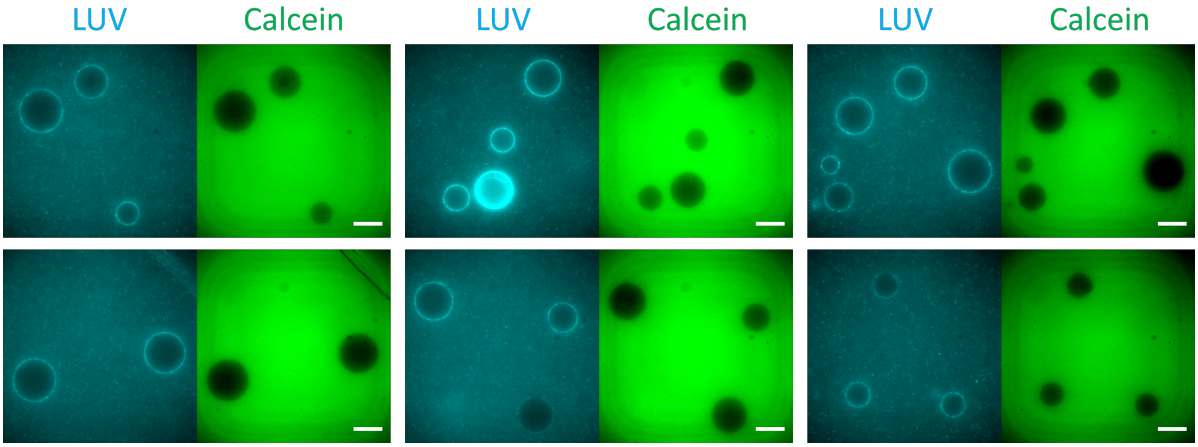


Figure C.4 Performing the calcein-cobalt content mixing assay to detect DNA-mediated vesicle fusion. The sample shows a higher calcein background signal and higher GUV density compared to the charge-based experiment (Figure C.3), which we think is due to the single washing step performed for this experiment. Vesicles show a relatively low level of GUV-LUV binding, and no single fused vesicle was observed. Scale bars indicate 20 µm.

C.2.2 R18 self-quenching assay

Another fusion method we tested was based on the self-quenching characteristics of the dye R18. We performed two approaches to incorporate R18 in GUV membranes: through direct incorporation (Figure C.5a) or by externally adding different amounts of R18 to GUVs after vesicle formation (Figure C.5b-d). A high R18 background signal was observed for the samples were R18 was added externally. We therefore assumed that the low R18 intensity values found for these vesicles was not caused by self-quenching, but represented a low level of R18 incorporation. We therefore used vesicles where R18 was directly incorporated to detect GUV-LUV fusion.

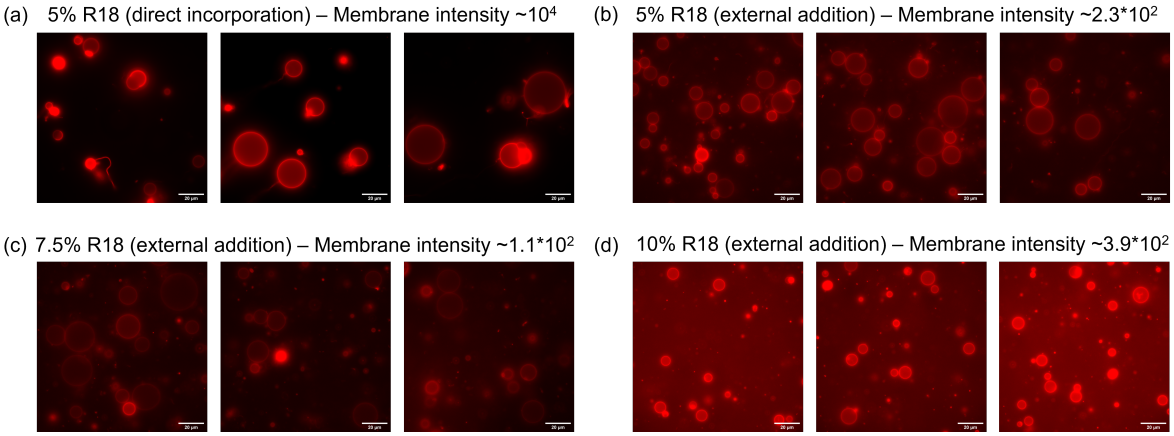


Figure C.5 Incorporation of R18 in GUVs by direct incorporation (a) or by externally adding 5 mol% (b), 7.5 mol% (c), or 10 mol% R18 (d) to GUVs after vesicle formation. Membrane intensities were estimated through intensity profile plotes, where averages of ~5 vesicles per sample were taken and the background signal was subtracted. Scale bars indicate 20 µm.

The results of the R18 self-quenching assay to detect DNA-mediated fusion are displayed in Figure C.6. Here, we hypothesised that GUVs that underwent GUV-LUV fusion would show a higher R18 signal, caused by dilution of the R18 dye. However, we observed great variety in R18 signal on the GUV membrane both for GUVs that were mixed with LUVs with or without DNA. Based on these qualitative results, we were therefore not able to determine if fusion occurred. Fusion can possibly be detected by quantifying the data: if a higher R18 signal will be found on the GUV membranes that were exposed to LUVs with DNA compared to GUVs mixed with LUVs without DNA, this could indicate GUV-LUV fusion. However, it is questionable if sufficient LUVs have fused with GUVs to detect a significant increase in R18 signal on the GUV membrane. This would have required many fusion events because of the dramatic difference in surface area between GUVs and LUVs.

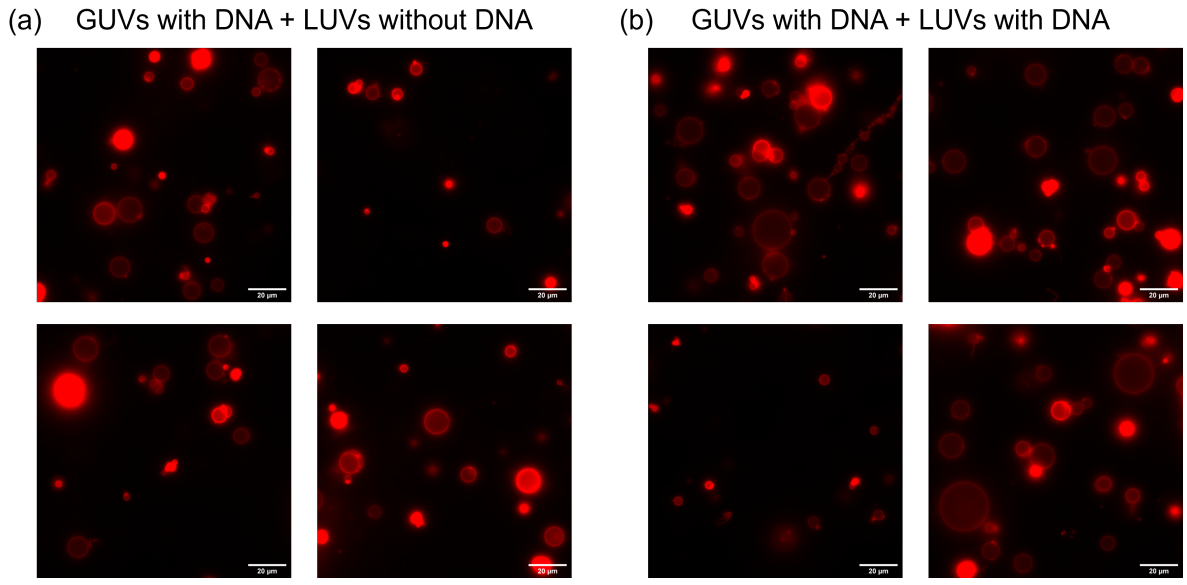


Figure C.6 Testing the R18 self-quenching assay for DNA-mediated vesicle fusion. R18 was directly incorporated in GUVs with DNA, that were mixed with LUVs with or without DNA. Scale bars indicate 20 µm.

C.2.3 Streptavidin-biotin fusion assay

We proposed a fusion assay based on the strong binding affinity between streptavidin and biotin (Subsection C.1.3). We found that the streptavidin was successfully encapsulated in GUVs (Figure C.7). We observed some heterogeneity in internal streptavidin signal, which could indicate that streptavidin was not encapsulated as efficiently in each GUV. The difference in internal streptavidin signal could

also be due to imaged GUVs being at different z-heights. We incubated GUVs with Proteinase K to degrade external streptavidin. Earlier work showed that Proteinase K activity increases at 37℃ compared to room temperature [236]. However, we were uncertain about the stability of DNA incorporated at 37℃. We therefore incubated GUVs with Proteinase K either at room temperature for 30 minutes (Figure C.7a) or at 37℃ for one hour (Figure C.7b). Despite the addition of Proteinase K, we observed a streptavidin background signal for both samples. We hypothesise that Proteinase K did not fully degrade streptavidin, and/or only degraded the protein but not its fluorescent tag.

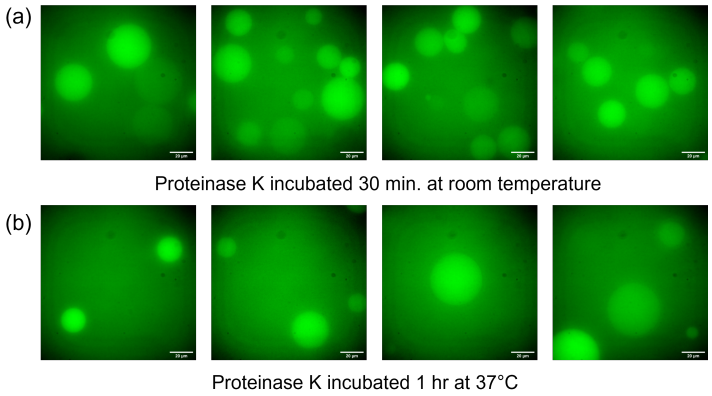


Figure C.7 Encapsulation of fluorescent streptavidin in GUVs. Proteinase K was incubated for 30 min. at room temperature (a) or for 1 hr at 37°C (b). Scale bars indicate 20 µm.

We added LUVs with DNA to GUVs with DNA encapsulated with streptavidin. Here, we found high levels of GUV-LUV binding for the GUVs that were incubated at room temperature (Figure C.8a), but little to no GUV-LUV binding for GUVs incubated at 37℃ (Figure C.8b). Next to this, we observed clear streptavidin localisation on the membranes of GUVs incubated at room temperature, whereas this localisation was lacking for the other GUV sample. Localisation of streptavidin on the GUV membrane could indicate GUV-LUV fusion. However, the fact that the streptavidin signal is patchy, as well as its resemblance with the LUV signal, suggests that streptavidin localisation on the GUV membrane was at least partly due to external streptavidin binding to LUVs. Here, we are unsure if these LUVs genuinely fused with GUVs, or if they were only bound to the GUV membrane. By including a control in a future experiment, where streptavidin is not encapsulated in GUVs but only added externally, it could be assessed if external streptavidin indeed contributes to streptavidin localisation on the GUV membrane.

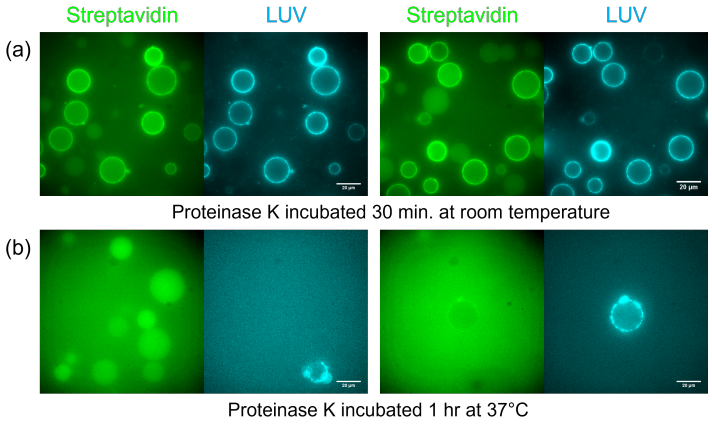


Figure C.8 Testing the streptavidin-biotin fusion assay for DNA-mediated GUV-LUV fusion. Proteinase K was incubated for 30 min. at room temperature (a) or for 1 hr at 37°C (b). Sample (a) showed clear GUV-LUV binding and streptavidin localisation on the GUV membrane, that could indicate GUV-LUV fusion, but which could also be the result of external streptavidin binding to bound but unfused LUVs. Sample (b) showed little to no GUV-LUV binding and streptavidin localisation. Scale bars indicate 20 µm.

We observed little to no GUV-LUV binding and streptavidin localisation on the membranes of GUVs incubated at 37℃ (Figure C.8b). We hypothesised that DNA might properly incorporate at this temperature. Therefore, we assessed the ability to incorporate DNA into GUVs at 37℃ at different steps of the DNA incorporation protocol (Figure C.9a-c, see Table C.1 for the details of the different samples). We also added a sample where DNA was incorporated at room temperature (Figure C.8d). We observed variety in DNA signal before mixing, but similar results after mixing. The fact that samples displayed in Sub-figures C.9ab already showed a higher DNA signal before mixing suggests that DNA incorporation without mixing is accelerated at 37℃, since DNA was incorporated at this temperature for these samples. All samples showed comparable levels of DNA incorporation after mixing. We therefore conclude that DNA incorporation at 37℃ is comparable to DNA incorporation at room temperature and is independent of the time step of adding DNA.

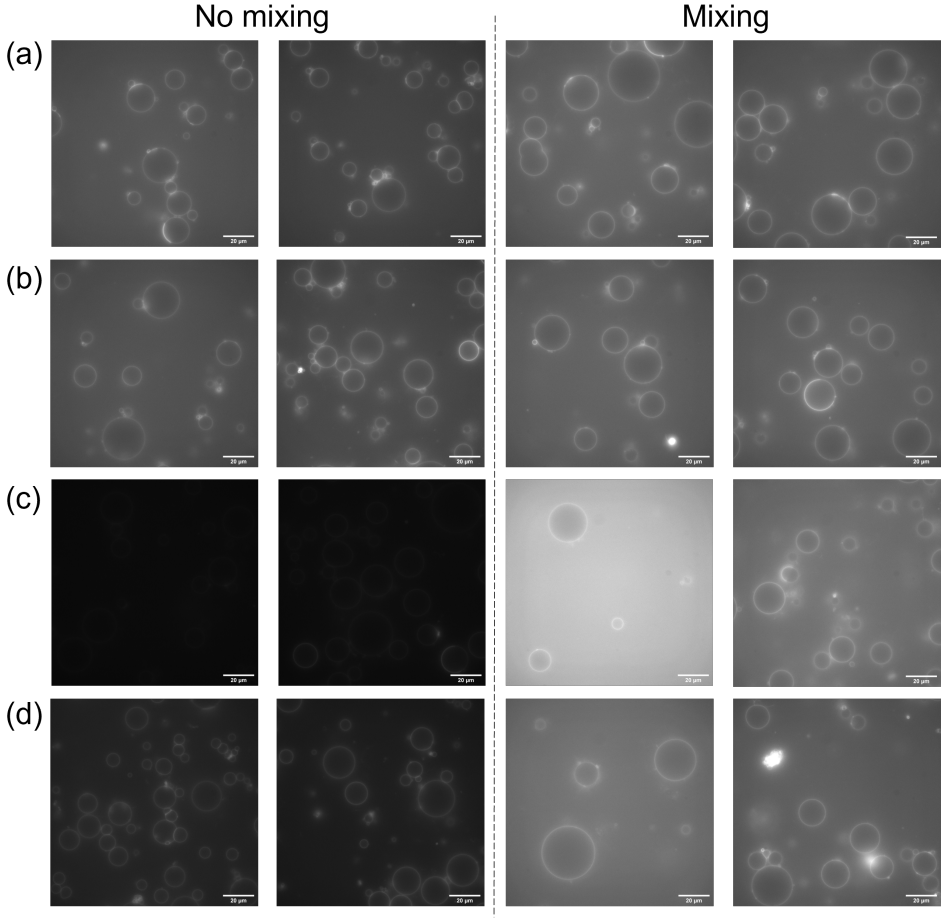


Figure C.9 Assessing the ability of DNA to incorporate into GUVs at 37°C. DNA was either added one hour before (a, sample 1), two minutes before (b, sample 2), or 30 minutes after incubation at 37°C. (c, sample 3). DNA was incorporated at room temperature for one other sample (d, sample 4). DNA incubation times are displayed in Table C.1. After mixing, all samples showed similar levels of DNA incorporation.

C.2.4 His-nickel fusion assay

In Subsection C.1.4, we proposed an adapted streptavidin-biotin fusion assay, where the two binding compounds are replaced with His-GFP and nickelated lipids. We assessed the ability of Proteinase K to degrade His-GFP at room temperature and at 37℃ with a bulk fluorescence experiment. Although we expected a lower fluorescent signal for samples with Proteinase K through the degradation of His-GFP by this compound, we generally measured a higher fluorescent signal for samples with Proteinase K (Figure C.10). This high level of fluorescence was not found when Proteinase K was measured in absence of His-GFP, indicating that the fluorescent signal was not caused by autofluorescence of Proteinase K. Earlier work showed that Proteinase K activity is further optimised at a higher temperature

of 45 ℃ [236]. Although we do not expect that this relatively small temperature difference fully explains the trend we observed, it could be worthwhile to assess if His-GFP is more effectively degraded by Proteinase K at this higher temperature. If it turns out that His-GPF is degraded effectively at 45 ℃, it should be assessed if DNA is still incorporated at this higher temperature to allow for the development of the His-nickel fusion assay.

Gain = 110 (optimal); T = 35.6 °C; Ex. = 395 nm	20 °C	37 °C
GFP + ProK	38074	23747.3333
GFP without ProK	14726	20099

Gain = 100; T = 21.7 °C; Ex. = 395 nm	20 °C	37 °C
GFP + ProK	18158.66667	10088
GFP without ProK	6183.666667	9369

Gain = 100; T = 21.7 °C; Ex. = 475 nm	20 °C	37 °C
GFP + ProK	7079.667	3818.667
GFP without ProK	2446.667	3681

Gain = 115; T = 20.4 °C; Ex. = 395 nm	20 °C	37 °C
GFP + ProK	50488.33333	26171
GFP without ProK	17031	27468.6667

Gain = 115; T = 20.4 °C; Ex. = 475 nm	20 °C	37 °C
GFP + ProK	17427	8644
GFP without ProK	5884	10316

Gain = 115; T = 20.4 °C; Ex. = 395 nm	20 °C
Buffer + ProK	121
Buffer without ProK	246.6666667

Gain = 115; T = 20.4 °C; Ex. = 395 nm	20 °C
Buffer + ProK	95
Buffer without ProK	269

Figure C.10 Assessing the ability of Proteinase K to degrade His-GFP at room temperature and 37°C. Values indicate the fluorescence that was measured by the plate reader at different gains and excitation wavelengths of 395 nm (blue) and 475 nm (green). Contrary to what we would expect, we generally measured a higher fluorescent signal in the presence of Proteinase K.

D

Micropipette aspiration experiments

D.1 Materials and methods

D.1.1 Micropipette fabrication

Micropipettes with a tip diameter of 5-10 µm were prepared for micropipette aspiration (Section D.1.3) and local SUV/LUV injection experiments (Section D.1.4). These micropipettes were fabricated by first cutting a borsilicate glass capillary (30-0016, Harvard Apparatus, outside diameter = 1.0 mm, inside diameter = 0.58 mm, length = 100 mm) with the laser-based micropipette puller (Model P-2000, Sutter Instrument, Heat = 350, Fil = 4, Vel = 50, Del = 255, Pul = 150). The micropipette pulling resulted in glass capillaries with a closed tip of a few microns wide. To open the tip and make the capillary hollow again, the tip was temporarily put in a droplet of liquid glass. This resulted in an open tip of a few microns wide with an irregular and sharp ending. To acquire the desired tip size and make the ending blunt, the tip was again temporarily put in the droplet of liquid glass. Since the tip was open at this step, glass entered the tip through capillary forces. When the tip was brought out of the liquid glass, it was cut at the glass-air interface inside the capillary, resulting in a blunt end of the desired size [237]. The micropipette tip was fabricated while observing it with a light microscope (MF-900 Microforge, © NARISHIGE Group) with a 35x objective lens and a 15x eyepiece. The tip diameter could be determined with a small ruler in the eyepiece of the microscope.

D.1.2 Chamber preparation

Chambers for micropipette experiments were prepared by cutting a cover glass (24x50 mm, No. 1.5H, Paul Marienfeld GmbH & Co. KG) in two pieces of approximately 10x50 and 14x50 mm. This was done using an engraving pen (Sigma-Aldrich), and the cutting was performed on a Harris cutting mat (Sigma-Aldrich). The glass slides were cleaned with ethanol and Milli-Q water and dried under a stream of nitrogen gas. Then, the glass slides were fixed on the opposite sides of a metal holder with beeswax (Beckman Coulter), creating a microscopy chamber in between the two glass slides. The chamber was passivated for at least 15 minutes by adding 50-150 µL 1 mg/mL beta-casein in a solution of 200 mM glucose and 10 mM Tris (pH 7.4). Here, we tried to keep the beta-casein droplet in the middle of the chamber without the droplet touching the edges. If the droplet did touch the edges, this could lead to more evaporation during the experiment which might influence the results. The micropipette was put in the beta-casein droplet to passivate that as well. After passivation, the beta-casein was removed with the tip of a tissue, and the chamber was flushed at least two times with OBS. This was done by adding 50-150 µL OBS (equal to the amount of beta-casein that was added) and removing this with the tip of a tissue as well. After flushing, (quenching) OBS and vesicles were added, where the type and amount of OBS and vesicles added depends on the experiment performed.

D.1.3 Micropipette aspiration

Micropipette aspiration (MPA) experiments were performed with the aim to develop a set-up that is able to monitor membrane growth for single vesicles by looking at their two-dimensional projection. To achieve this, it should first be possible to measure a constant membrane area when no membrane growth is expected. This can be a great challenge, since small amounts of evaporation or changes in pressure can influence the measured membrane area. The first MPA trial was done with 0.1% Atto655 GUVs that were prepared in an adjusted GUV swelling buffer containing no KCl, 200 mM sucrose

and 10 mM Tris (pH 7.4). The adjusted GUV swelling buffer was used to match the osmolarity of the beta-casein used for passivation (Subsection D.1.2). The micropipette was filled with 10 µL adjusted OBS consisting of 200 mM glucose and 10 mM Tris (pH 7.4). Before the micropipette was placed in its holder, any air bubbles in the tube attached to the holder were removed. Once the pipette was fixed in its holder, air in the tip of the pipette was removed using the plunger of the MPA set-up.

After chamber passivation and flushing, 80 µL of the same adjusted OBS and 10 µL GUVs were added to the chamber. A waiting step of 10 minutes was performed to allow for some evaporation, resulting in more fluctuating vesicles that are easier to grab with the micropipette. Then, the vesicle solution was sealed with mineral oil (Sigma-Aldrich) to prevent further evaporation. Here, it was ensured that the mineral oil did not touch the edges of the chamber, since this could decrease the efficiency of the mineral oil to prevent evaporation. The zero pressure of the MPA set-up was found by observing no flow in- or outward of the micropipette. Next, multiple vesicles were consecutively aspired with the MPA set-up by applying a negative pressure. The vesicles were observed over time for 30 seconds, where 10 frames per second (fps) were imaged for the first 3 seconds, and 1 fps for the remaining time. Additionally, the vesicles were observed under different amounts of membrane tension by adjusting the magnitude of the negative pressure. The GUV-0.1%Atto655 optical configuration was used to detect vesicles, where a LED intensity of 5% was used. The evolution of membrane area was determined with a pre-written Python script developed by Lennard van Buren.

An additional MPA trial was performed with 0.1% Atto655 GUVs formed by electroformation [85]. This experiment was performed in the same way as the first trial, except for a few exceptions. The first exception is that normal OBS was present in the micropipette instead of the adjusted OBS. Next to this, one vesicle was observed for 30 minutes instead of 30 seconds (0.1 fps for the first 3 seconds, 1 frame per minute for the remaining time). With this trial, it could thus be determined if membrane area remains constant for an extended period of time. Finally, a LED intensity of 20% was used to detect the GUVs.

D.1.4 Local SUV/LUV injection

Local SUV/LUV injection experiments were performed with fusion mechanisms based on both DNA and charge (only results for DNA-mediated fusion are shown). The charge-based experiments were performed with 0.1% Atto655 GUVs, 30 nm 20% TAP SUVs (one week old) and 100 nm 20% TAP LUVs (fresh). For the experiment with 30 nm SUVs, 10 µL GUVs were added to 100 µL OBS. For the experiment with 100 nm LUVs, 14 µL GUVs were added to 140 µL OBS. For the DNA-based experiment, 0.5% Atto488 GUVs and 200 nm Atto655 LUVs were used. In this case, 15 µL GUVs were added to 135 µL quenching OBS. For all local injection experiments, 10 µL SUV/LUV vesicle solution was added to a micropipette. Before the micropipette was inserted into the injector (CellTram® 4r Oil, Eppendorf), it was ensured that any air bubbles in the tube attached to the injector were removed. Once the micropipette was inserted, SUVs or LUVs were locally injected into the sample by applying a positive pressure. For the charge-based experiments, the GUV and SUV/LUV signals were measured over time using the GUV-0.1%Atto655 and SUV/LUV-RhoPE optical configurations. Here, a LED intensity of 10% was used to detect GUVs. For the DNA-based experiment, the GUV, LUV and HPTS signals were measured over time using the GUV-0.5%Atto488, SUV/LUV-Atto655 and HPTS optical configurations (Table 3.6).

D.2 Results

D.2.1 Micropipette aspiration

We performed multiple experiments to measure the membrane area evolution of GUVs aspirated under constant pressure (Figure D.1). Here, the distances in mm correspond to the application of negative pressure, where more pressure was applied for larger distances. This can be observed from the tongue of the GUVs (the part that is aspirated into the micropipette), that became longer for higher pressures. The graphs show the membrane area evolution over time, where a difference in detected membrane area of 2% is accepted as a constant membrane area. In Figures D.1ab, the GUVs remained in this

tolerance region for at least 3 minutes in most cases.

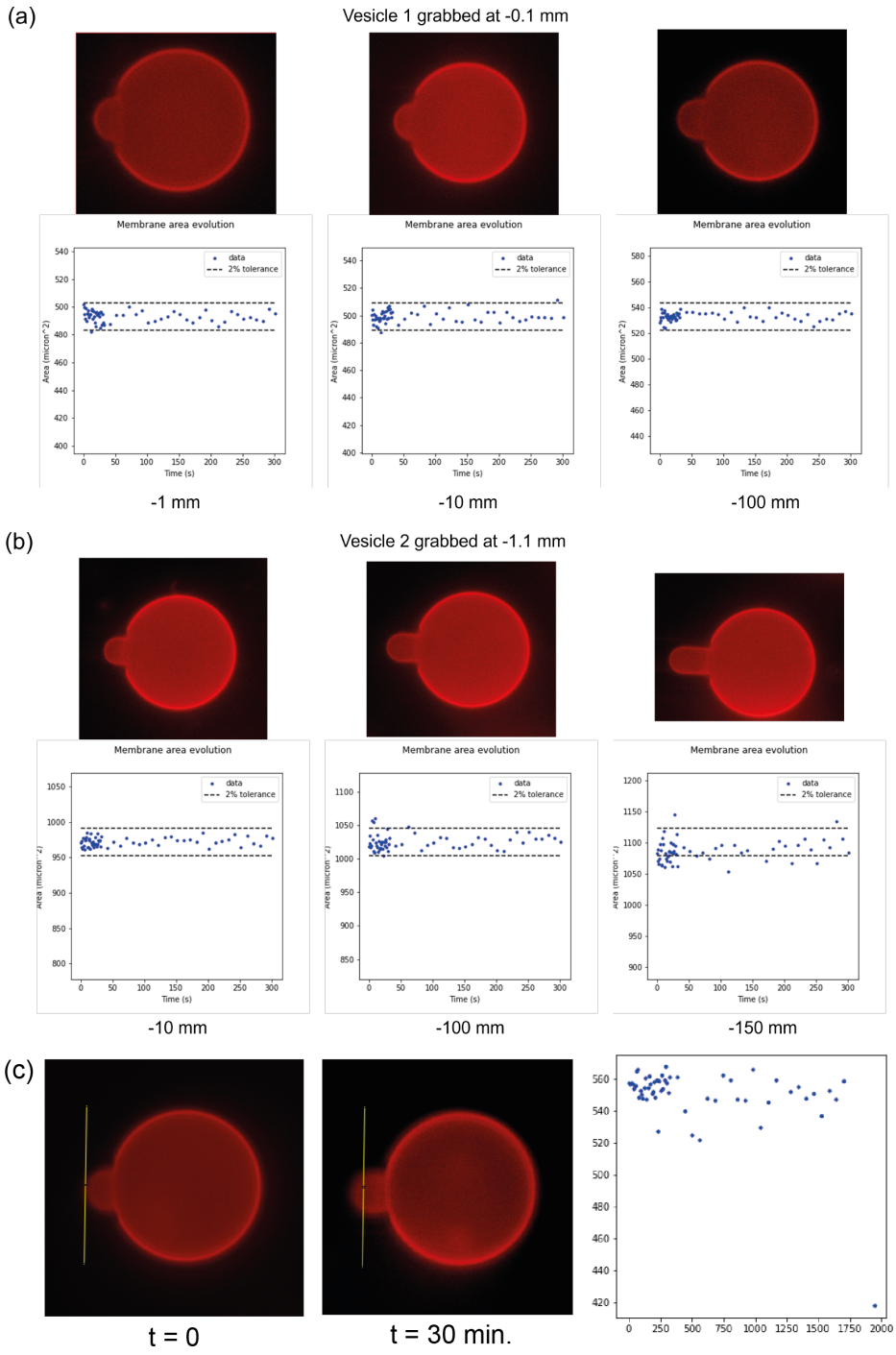


Figure D.1 Assessing membrane area evolution for GUVs trapped in an MPA set-up under constant pressure. GUVs (a) and (b) correspond to the first trial described in Subsection D.1.3 and were formed with gel-assisted swelling. The GUVs show a constant membrane area for different negative pressures in the majority of cases. GUV (c) corresponds to the second trial described in Subsection D.1.3 and was formed by electroformation. A relatively constant membrane area was measured for 30 minutes, where the final outlier is expected to be caused by water evaporation on the water objective.

To measure changes in membrane area upon vesicle fusion, one possibly requires longer timescales. We therefore assessed membrane area evolution under constant pressure for 30 minutes (Figure D.1c). Here, we found a relatively constant membrane area as well. The vertical line at the two different time points remained in the same place, showing that the tongue of the GUV increased a little

in size. We believe that the data point at the latest time point, showing a dramatically lower membrane area, was caused by evaporation of water on the water objective. This indicates that water should be added to the objective during extended MPA experiments.

D.2.2 Local SUV/LUV injection

We performed experiments where we locally injected SUVs or LUVs to GUVs to allow for the live observation of vesicle binding. In Figure D.2, an overview of this experiment is provided for DNA-mediated fusion. In this case, LUVs were locally injected with a micropipette to GUVS in solution (a), and imaged in the GUV and LUV channel (b). With this approach, we were able to observe and measure an increase in LUV signal on the GUV membrane over time, while we measured a constant GUV signal (c and d). When combining this approach in future experiments with the MPA experiments described in Subsection D.2.1, one could measure the membrane area evolution of single GUVs upon GUV-LUV fusion. Here, it would be beneficial to combine the approach with a method to observe live vesicle fusion, for example by further developing the His-nickel fusion assay (Subsection C.2.4), or by preventing the membrane anchor used for DNA insertion to desorb (Subsection 5.8.3).

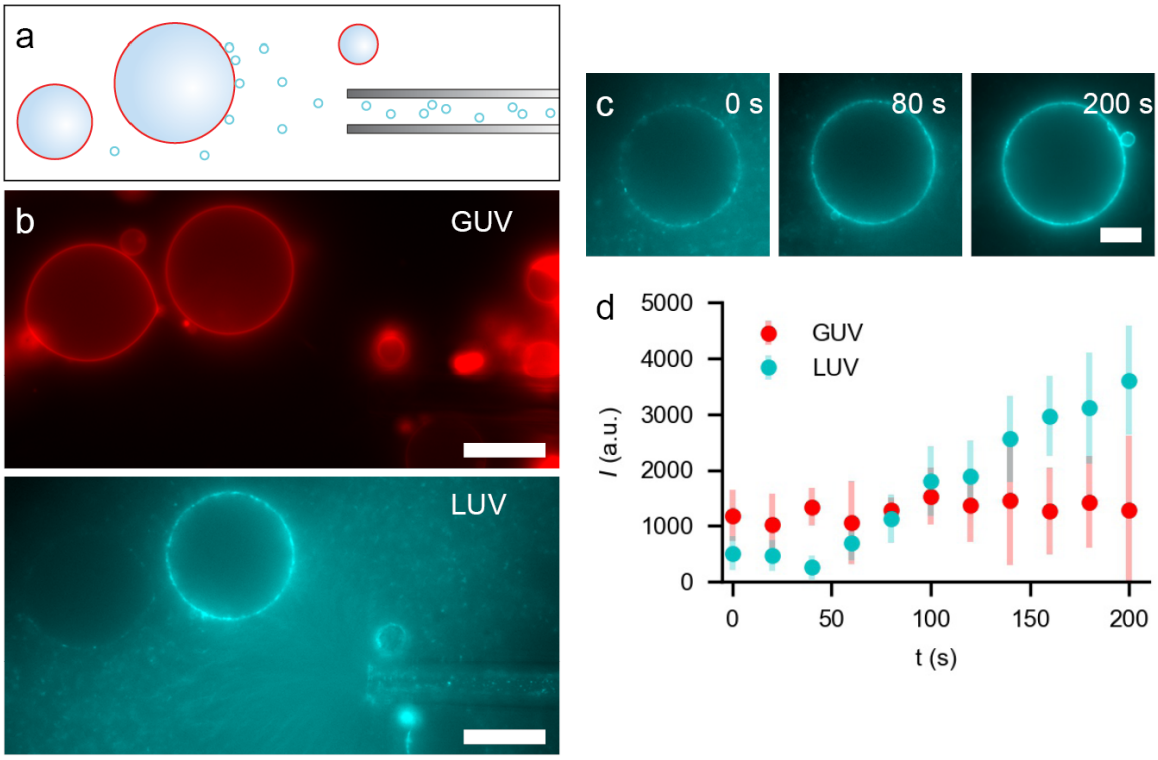


Figure D.2 Local injection of LUVs to GUVs to observe live GUV-LUV binding. (a) Schematic representation of the set-up. (b) Vesicles were imaged with epifluorescence microscopy in the GUV and LUV channel, where the micropipette is visible in the right-bottom corner. (c) Through DNA-mediated binding, LUVs localise on the GUV membrane over time. (d) Quantification of the GUV and LUV intensity over time of the GUV membrane displayed in (c). Membrane intensities were determined as described in Section 3.12, data points indicate background-subtracted GUV/LUV intensity, error bars represent standard deviations. Scale bars indicate 20 µm. Figure made by Lennard van Buren.

E

Interview protocols

Below, an overview of the interview protocols is provided. The interview protocols are in Dutch, since this was the main language of the interviewer and interviewees.

E.1 Interview protocol Usai and Dogterom

Bedrijven

- Jullie hebben al aangegeven dat er al wat contacten zijn met bedrijven, maar
  - Wat zijn jullie wensen op de lange termijn? (Een public-private partnership zoals BE-Basic (biotech PPP? Samenwerking met valorisatiecentrum hiervoor?)
  - Wat hebben jullie daarvoor nodig?
  - Met bedrijven uit wat voor sectoren zouden jullie samenwerkingen willen?
  - Proposal Europa, funding voor onderzoek? Ook voor opzetten samenwerkingen met industrie?

Huidig contact

- Hoe zijn de huidige contacten met bedrijven tot stand gekomen?
  - Vooral uit bestaande contacten?
- Het viel me op dat er redelijk veel contact is met chemiebedrijven, is hier een bepaalde reden voor (bestaande contacten?)?
- Hoe ziet het contact met deze bedrijven eruit?
  - Is de huidige vorm van samenwerking puur de aangetoonde interesse van bedrijven, of zijn er ook al bedrijven die bijv. een intentieverklaring hebben ondertekend?
  - Wie vanuit SyCell heeft er allemaal contact met bedrijven?
  - Hoe vaak wordt er gesproken?
  - Hoe zien die meetings eruit? Wordt er met één/meerdere personen uit een bedrijf gesproken, of meerdere bedrijven tegelijkertijd (in brainstormsessies)?
- Weten jullie of de bedrijven zelf ook al onderzoek doen naar synthetische cellen? Of geven ze vooral aan in gesprekken wat ze interessant onderzoek zouden vinden? (Stefania gaf via de mail aan dat de bedrijven hebben aangegeven dat ze geïnteresseerd zijn in onderzoek naar synthetische cellen, maar ik vraag me af hoe dit er in de praktijk uitziet)
- Zou het voor mij mogelijk zijn om eens aan te sluiten bij zo'n brainstormsessie?

Nieuwe samenwerkingen

- Op welke manieren wordt er geprobeerd om meer contact te krijgen met bedrijven?
  - Is hiervoor strategisch overleg tussen jullie en de verschillende PI's van BaSyC/SyCell, of is dit iets waar jullie vooral individueel mee bezig zijn?

  - Hoe krijg je bedrijven geïnteresseerd in synthetische cellen?

- Denken jullie dat eventuele concurrentie tussen bedrijven het aangaan van samenwerkingen met bedrijven beïnvloedt? (Bedrijven zouden bijv. eerder een samenwerking aan kunnen gaan omdat ze bang dat ze anders “de boot missen”, maar dit zou het aangaan van extra samenwerkingen ook weer kunnen verhinderen).
- Is er bepaalde regelgeving die het aangaan van samenwerkingen moeilijk maakt (met bedrijven, maar ook in de algemene zin van het woord. In het geval van bacteriofagen is dit het geval)?

Lobby in Brussel

- Hoe ziet de lobby in Brussel er in de praktijk uit?
  - Lobby alleen voor multidisciplinair instrument, of ook synthetic cell op de kaart zetten / andere redenen?
  - Hoe actief wordt deze lobby uitgevoerd?
  - Wie zijn hier allemaal bij betrokken? (Alleen mensen van SyCell, of ook bedrijven/de overheid/een contactpersoon in Brussel?)
  - Wat zijn de plannen als de lobby succesvol is? In hoeverre zijn deze plannen concreet? Stefania heeft eerder eens aangegeven dat het ontbreken van Europese funding één van de redenen dat er nog geen formele samenwerkingen zijn met bedrijven. Zouden bedrijven niet ook mee kunnen doen met een fundingaanvraag?
- Kijken jullie ook naar succesvolle casussen van onderzoekers die samenwerkingen zijn aangegaan met industrie/de overheid om hier van te leren?

Overheid

- Wat voor contact is er met de Nederlandse overheid? (Doel van de vraag: Te weten te komen wat voor contact er is met de overheid, en of de overheid bepaalde intenties/belangen/wensen heeft op het gebied van de synthetic cell)
  - Gaat het contact met de NWO puur over het functioneren van BaSyC, of zijn er nog andere vormen van contact?
  - Heeft de overheid nog een bepaalde rol in de lobby in Brussel?
  - Wat voor overheidsinstelling zou interessant zijn om mee samen te werken? Waarom?

Onderzoekers

- Van Gijsje hoorde ik dat er niet per se heel veel concurrentie is tussen de onderzoeksgroepen van SyCell (het is een groot project en ze werken complementair aan elkaar), hoe zit dit met andere synthetic cell samenwerkingen wereldwijd (buil-a-cell, samenwerking in Japan?)?

Responsible research and innovation

- Eén van de concepten die ik onderzoek voor mijn thesis is “Responsible research and innovation”.
- Zijn jullie bekend met dit concept?
- Speelt dit (of onderdelen van RRI, zoals het betrekken van de maatschappij, inclusiviteit en duurzaamheid) een rol binnen jullie werk als onderzoeker/strategisch adviseur/program manager?
  - Hoe werkt dit in de praktijk? (Voorbeelden?)

- Speelt dit een rol binnen het aangaan van samenwerkingen voor SyCell?
  - Hoe werkt dit in de praktijk? (Voorbeelden?)
- Artikel gelezen over hoe RRI wordt toegepast in BrisSynBio, drie rollen voor synthetic biologists: epistemics (kennis genereren), pragmatic constructors (synthetische biologie om grote problemen op te lossen en nieuwe producten te maken), or committed engineers (engineering principes toepassen om de biologie te kunnen manipuleren op een betrouwbare en voorspelbare manier). Hoe zien jullie de rol van de synthetic biologist?

Afsluitende vragen

- Wat zijn jullie dromen voor BaSyC/de synthetic cell initiative?

E.2 Interview protocol Bovenberg

Mezelf voorstellen. Bedankt dat u de tijd heeft voor een gesprek over de samenwerkingen tussen BaSyC en de industrie. Vindt u het goed als ik het gesprek opneem? Aan het begin van de opname zal ik vragen of u het goed vindt dat ik inzichten en eventuele quotes uit het gesprek mag gebruiken in mijn thesis na uw goedkeuring. Deze quotes kunnen ook geanonimiseerd worden als u dit wenst.

Introductie

- Zou u wat over uw eigen achtergrond kunnen vertellen? Wat heeft u zoal uitgevoerd in uw carrière en hoe bent u op uw huidige posities terecht gekomen?

BaSyC

- Hoe bent u bij BaSyC terecht gekomen?
- Wat is uw rol binnen het advisory board van BaSyC?
- Hoe ziet dit advies geven er in de praktijk uit?
  - Hoe vaak overlegt u binnen het advisory board?
  - Hoe vaak geeft u advies aan de steering committee (gebeurt dit op vaste momenten, of varieert dit?)?
- Bent u ook betrokken bij het Europese Synthetic Cell initiative?
  - Wat is uw rol hier?
  - Hoe verschilt deze samenwerking met de Nederlandse samenwerking?
- Ik las in een interview met u dat u heeft meegeholpen aan het opzetten van meerdere public-private partnerships. Zou u wat meer over het opzetten van deze PPPs kunnen vertellen en hoe u de inzichten van toen nu toepast op BaSyC/SyCell?
- Is zo'n PPP volgens u ook waar BaSyC/Sycell op de lange termijn heen moet, of een andere vorm van samenwerking (een innovation ecosystem)?
  - Op nationaal/Europees niveau?
  - Met wat voor bedrijven (uit welke sectoren) ziet u zo'n samenwerking voor u?
  - Wat is de rol van de overheid? (Zijn er bepaalde overheidsinstanties waarmee het interessant is om samen te werken?)
- Hoe verschilt BaSyC/SyCell van de PPPs waar u eerder aan meegewerkt hebt?

- Ik las in hetzelfde interview dat “early involvement” essentieel is. Zou u dit verder kunnen toelichten?
- Tegen wat voor uitdagingen en onzekerheden loopt u aan in het aangaan van samenwerkingen met de industrie?
  - Zowel voor BaSyC in het algemeen als persoonlijk (is het moeilijk om een bedrijf als DSM mee te krijgen als het iets is waar u persoonlijk erg in gelooft)?
  - Hoe spelen verschillende perspectieven/verwachtingen van verschillende stakeholders hierin een rol?

Innovation models & RRI

- Er zijn meerdere modellen die een innovatieproces omschrijven. Bij klassieke lineaire modellen wordt een wetenschappelijke ontdekking omgezet in een innovatie, zonder per se te weten of de maatschappij hier behoefte aan heeft. In andere modellen die later ontwikkeld zijn wordt er gekeken vanuit een probleem, wordt er een technologie gezocht om dit probleem op te lossen en wordt hierin ook naar de rol van de industrie gekeken. In het interview dat ik gelezen heb gaf u aan dat het moeilijk is de mogelijke toepassingen van een synthetische cel te voorspellen en dat deze vaak voortkomen uit onverwachte ontdekkingen. Denkt u dat het project hiermee meer bij het klassieke innovatiemodel past?
  - Als dit zo is: Hoe zorg je er in dit geval voor de maatschappij behoefte heeft aan de uiteindelijke toepassingen?
  - Zou het ook een combinatie van de twee innovatiemodellen kunnen zijn, waar er deels naar de mogelijke toepassingen van onverwachte resultaten wordt gekeken en deels naar het oplossen van maatschappelijke problemen?
- Gerelateerd aan “de behoefte van de maatschappij” is het concept responsible research and innovation (RRI). Bent u bekend met dit concept?
  - Zo ja: Wat betekent dit concept volgens u, en is er een manier waarop u dit toepast in uw dagelijkse bezigheden / het advies geven aan BaSyC?
  - Zo nee: betrekken van de maatschappij, op tijd betrekken van de industrie, duurzaamheid, inclusiviteit. Past u dit op een manier toe?
  - Tegen wat voor uitdagingen loopt u aan om RRI toe te passen?
- Een veelgenoemde kritiek op RRI is dat het moeilijk uitvoerbaar is in de praktijk. Eén van de gegeven redenen is dat werkdruk van onderzoekers al te hoog is/er al te veel taken zijn. Denkt u dat het het waard is als hier meer ruimte voor gecreëerd wordt (door het verplicht maken van bepaalde RRI-gerelateerde activiteiten, het extra belonen hiervan?)
  - Denkt u dat er andere manieren zijn om dit meer in de praktijk toe te passen?

E.3 Interview protocol Lohle and Jacobs

Mijn project

- Aangaan van samenwerkingen met de industrie in een fase van fundamenteel onderzoek → hoe doe je dit?
- Hoe kan je samenwerken in een innovatie-ecosysteem?

Achtergrond

Lohle

- Zou je wat meer over jezelf kunnen vertellen? Wat is je achtergrond, wat waren je werkzaamheden bij het valorisatiecentrum en wat is je huidige functie?
  - Ook bijgedragen aan BaSyC/SynCellEU?
  - Hoe blijf je als business relation manager op de hoogte van het onderzoek van de faculteit (wat voor contact met onderzoekers)? Hoe vertaal je dit naar bedrijven?

Jacobs

- Zou je wat meer over jezelf kunnen vertellen? Wat is je achtergrond, hoe ben je terecht gekomen bij je huidige functie en wat voor werkzaamheden verricht je voor het Innovation Impact centre?
  - Wat voor activiteiten heb je verricht op het gebied van technology transfer?
  - Hoe neem je lessen die je hebt geleerd uit het bedrijfsleven mee naar je huidige functie? Heb je hier voorbeelden van (wat voor plannen/visie heb je hiervoor)?

Bedrijven

- Hoe blijf je als business relation manager op de hoogte van het onderzoek van de faculteit (wat voor contact met onderzoekers)? Hoe vertaal je dit naar bedrijven?
- Hoe wordt het contact met bedrijven opgezet (vooral zelf benaderen/benaderd door bedrijven?)
  - Wat voor activiteiten komen hierbij kijken? (Lobbywerk/public relationship activiteiten?)
  - Hoe krijg je bedrijven geïnteresseerd voor onderzoek dat nog grotendeels fundamenteel is?
- Hoe wordt het contact met bedrijven onderhouden?
  - Wat voor activiteiten worden hiervoor georganiseerd?
- Wat voor soort bedrijven (vooral veel grote bedrijven of ook start-ups?)
  - Helpt het Innovation Impact Centre ook bij het vormen van start-ups, of is dit een taak van YES!Delft?
- Hoe ziet het contact met bedrijven er inhoudelijk uit (actief meedenken over innovaties/meer informeren over onderzoek?)

Innovaties

- Lineaire en non-lineaire innovatiemodellen. Wordt één van deze twee vooral gebruikt door het valorisatiecentrum, of een combinatie van de twee?
- Op de website staat dat het valorisatiecentrum zich inzet voor het voor het beschermen van vindingen met octrooien. Hoe gaat dit samen met het open samenwerken met bedrijven (open science/innovation)?
- Kan je een voorbeeld noemen van een succesvol innovatieproces waar het valorisatiecentrum aan heeft bijgedragen?
  - Wat maakte het een succesvol proces?
- Kan je een voorbeeld noemen van een minder succesvol innovatieproces?
  - Wat maakte het minder succesvol? Worden hier bepaalde lessen uitgetrokken?

Overheid

- Wat voor samenwerkingen zijn er met de overheid?
  - Met wat voor overheidsorganen?

- Hoe dragen deze samenwerkingen bij aan het realiseren van innovaties?

Responsible research and innovation (RRI)

- Ben je bekend met het begrip RRI (e.g. duurzaamheid, inclusiviteit, samenwerkingen tussen universiteiten en bedrijven vroeg beginnen en de maatschappij erbij betrekken, etc.)
  - Spelen elementen van RRI een rol in de werkzaamheden van het valorisatiecentrum (in het aangaan van samenwerkingen met bedrijven?)
  - Hoe zorg je dat RRI elementen in de praktijk worden toegepast (met wat voor werkzaamheden/activiteiten?)
  - Is er speciale aandacht voor bepaalde RRI elementen?

Overig

- Wil je nog iets kwijt over dit onderwerp waar we het niet over gehad hebben?

F

Provisional codes for interviews

Below, a list of provisional codes used to analyse the semi-structured interviews of Usai, Dogterom, and Bovenberg is provided.

Long-term vision

- Public-private partnership (PPP)
- Innovation ecosystem
- General long-term vision
- Difficulty in planning of long-term vision
- Early involvement

Collaborations with companies

- Formation of contacts with companies
- (Maintenance of) contacts with companies Type of companies/applications
- Type of collaboration with companies
- Research done by companies
- Competition between companies
- Role of regulations in forming collaborations with companies
- Joint/supported funding application
- Contact with companies
- Interest of companies

Lobby in Brussels

- Goal of the lobby
- Activities of the lobby
- People/organisations performing the lobby
- People/organisations encountered in the lobby
- European funding
- European coordination
- Pulled towards naming potential applications

- Arguments used in the lobby

Role of the government

- Current collaborations with the government/governmental organisations
- Potential interesting collaborations with the government/governmental organisations
- Activities performed by/with/for the government/governmental organisations

Researchers

- Competition between researchers
- Role of synthetic biologist

RRI

- RRI definitions/elements
- Practical implementation of RRI
- Role of RRI in starting collaborations
- Regulations

International advisory board

- Type of advice
- Frequency/form of advice given

SynCellEU

- Role at SynCellEU
- Difference with BaSyC

Innovation process

- Innovation models
- Formation of start-ups

Other

- Other collaborations
- Future panel

G

Code-to-article matrix

We constructed a code-to-article matrix to analyse what codes were discussed in what articles (Figure G.1, see next page). Here, the article numbers correspond to the article numbers of Table 8.1. Codes were either discussed extensively (dark green), discussed moderately (light green), mentioned but not discussed or related (yellow), or not discussed or mentioned (red). The distinction between discussed extensively and discussed moderately was made based on the number of times a code was mentioned and the depth of discussion. The code-to-article matrix is also available online, where citations and/or explanations related to the identification of the codes are provided.[1]

[1] Sheet 1 shows an overview of the articles corresponding to Table 8.1, sheet 2 shows the code-to-article matrix. Pieces of text in the code-to-article matrix were often directly copied from articles, so it mainly serves as on overview on how articles discuss certain concepts or themes. Quotation marks might be missing in some cases.

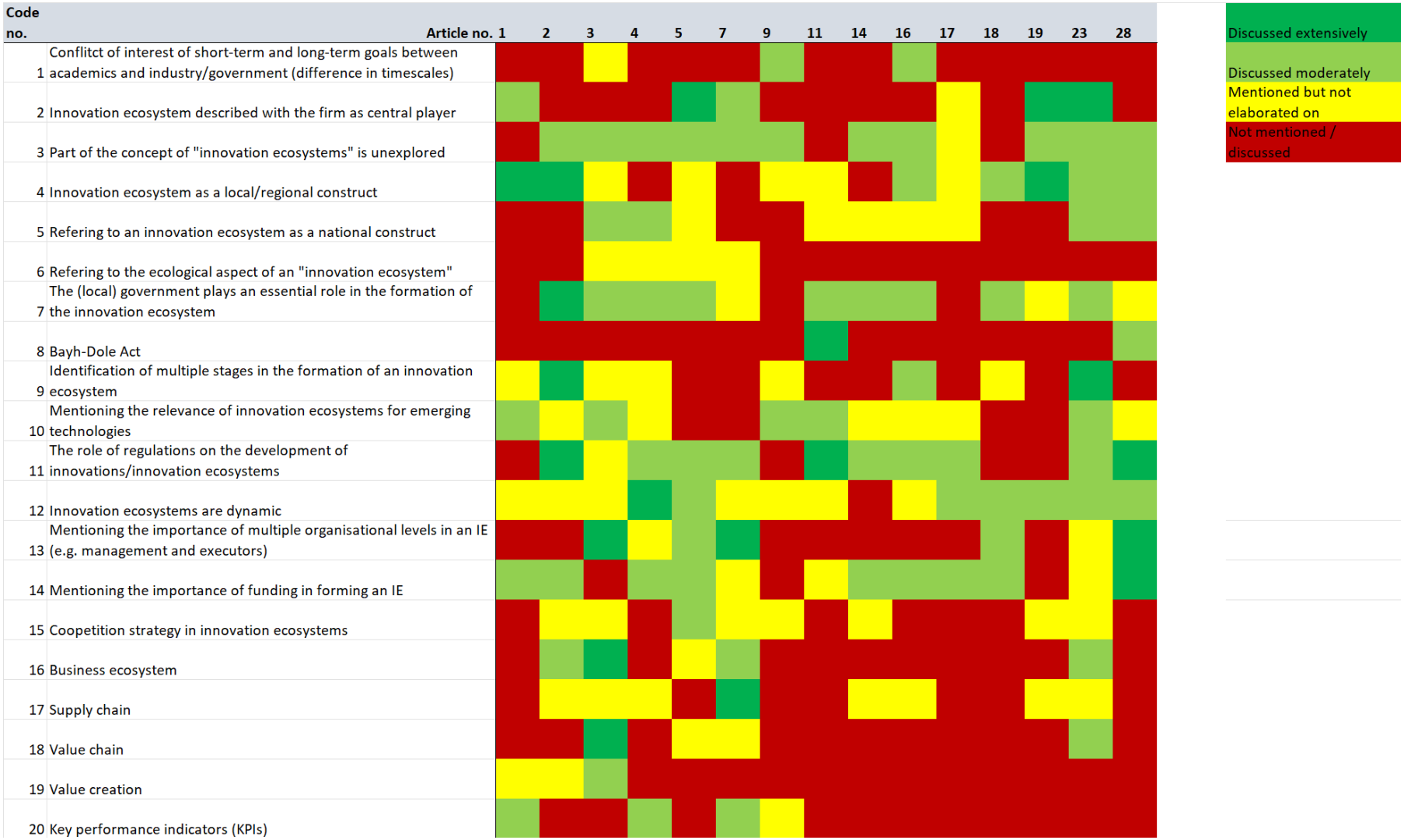


Figure G.1 Code-to-article matrix. Article numbers correspond to the article numbers provided in Table 8.1. Codes were either discussed extensively (dark green), discussed moderately (green), mentioned but not discussed or related (yellow), or not discussed or mentioned (red). Figure continues on next page.

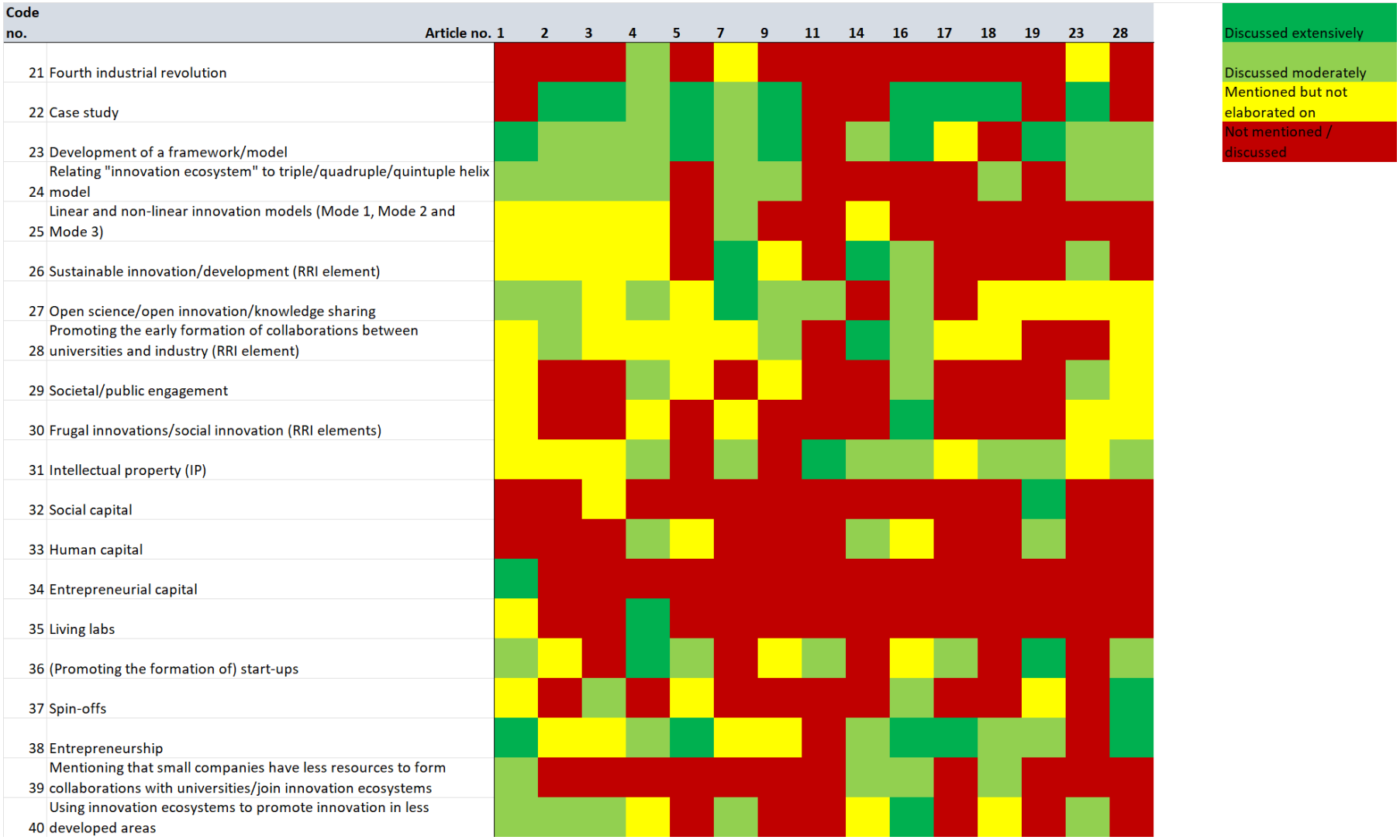


Figure C.1 Code-to-article matrix. Article numbers correspond to the article numbers provided in Table 8.1. Codes were either discussed extensively (dark green), discussed moderately (green), mentioned but not discussed or related (yellow), or not discussed or mentioned (red). Figure continues on next page.

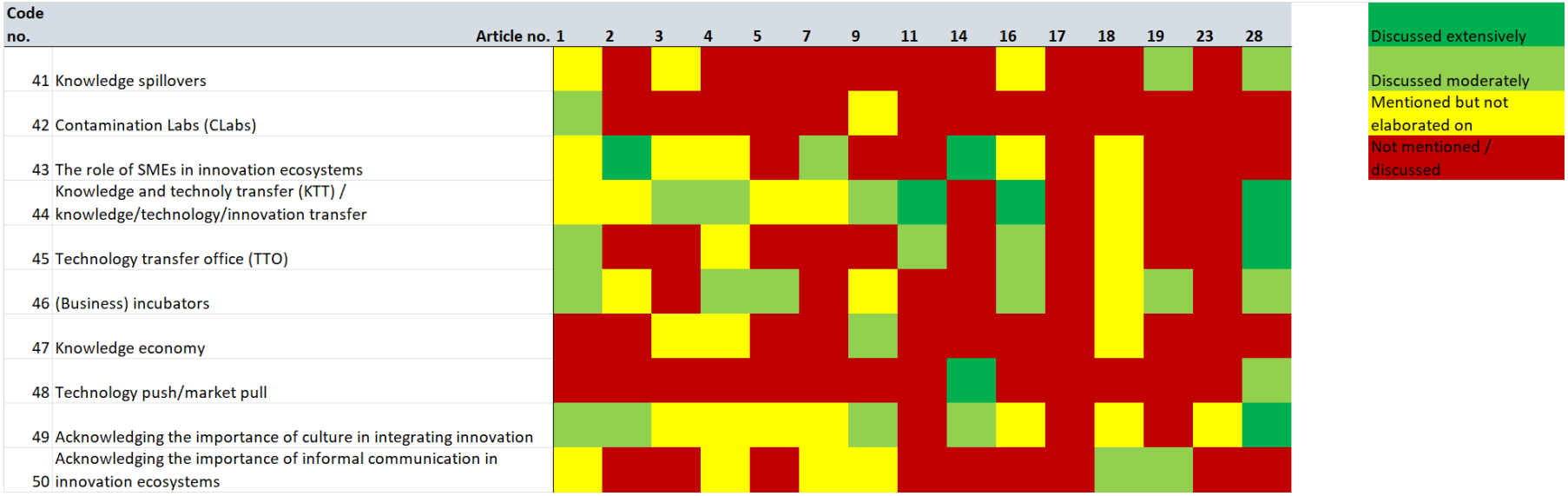


Figure C.1 Code-to-article matrix. Article numbers correspond to the article numbers provided in Table 8.1. Codes were either discussed extensively (dark green), discussed moderately (green), mentioned but not discussed or related (yellow), or not discussed or mentioned (red).

Bibliography

[1] University of Bristol. BrisSynBio. URL http://www.bristol.ac.uk/brissynbio/. Date accessed: 03-03-2022.

[2] Oxford English Dictionary. Meaning of human capital in English, 2022. URL https://www.lexico.com/definition/human_capital. Date accessed: 04-03-2022.

[3] Oxford English Dictionary. Definition of social capital in English, 2022. URL https://www.lexico.com/en/definition/social_capital. Date accessed: 04-03-2022.

[4] BaSyC. About BaSyC, 2021. URL https://www.basyc.nl/about-basyc/. Date accessed: 26-02-2021.

[5] Derek McCusker and Douglas R. Kellogg. Plasma membrane growth during the cell cycle: Unsolved mysteries and recent progress. *Current Opinion in Cell Biology*, 24(6), 2012. ISSN 09550674. doi: 10.1016/j.ceb.2012.10.008.

[6] S. Hadži and J. Lah. Origin of heat capacity increment in DNA folding: The hydration effect. *Biochimica et Biophysica Acta - General Subjects*, 1865(1), 2021. ISSN 18728006. doi: 10.1016/j.bbagen.2020.129774.

[7] Kevin Thurley, Lani F. Wu, and Steven J. Altschuler. Modeling Cell-to-Cell Communication Networks Using Response-Time Distributions. *Cell Systems*, 6(3), 2018. ISSN 24054720. doi: 10.1016/j.cels.2018.01.016.

[8] Brandon Ho, Anastasia Baryshnikova, and Grant W. Brown. Unification of Protein Abundance Datasets Yields a Quantitative Saccharomyces cerevisiae Proteome. *Cell Systems*, 6(2), 2018. ISSN 24054720. doi: 10.1016/j.cels.2017.12.004.

[9] Michelle G.J.L. Habets, Hub A.E. Zwart, and Rinie van Est. Why the Synthetic Cell Needs Democratic Governance. *Trends in Biotechnology*, 2020. ISSN 18793096. doi: 10.1016/j.tibtech.2020.11.006.

[10] Adam P. Arkin. Synthetic cell biology. *Current Opinion in Biotechnology*, 2001. ISSN 09581669. doi: 10.1016/S0958-1669(01)00273-7.

[11] Nathaniel J. Gaut and Katarzyna P. Adamala. Reconstituting Natural Cell Elements in Synthetic Cells. *Advanced Biology*, 5(3), 2021. ISSN 27010198. doi: 10.1002/adbi.202000188.

[12] Jonathan Garamella, David Garenne, and Vincent Noireaux. TXTL-based approach to synthetic cells. In *Methods in Enzymology*, volume 617. 2019. doi: 10.1016/bs.mie.2018.12.015.

[13] J. Dervaux, V. Noireaux, and A. J. Libchaber. Growth and instability of a phospholipid vesicle in a bath of fatty acids. *European Physical Journal Plus*, 132(6), 2017. ISSN 21905444. doi: 10.1140/epjp/i2017-11554-1.

[14] Jan Steinkühler, Roland L. Knorr, Ziliang Zhao, Tripta Bhatia, Solveig M. Bartelt, Seraphine Wegner, Rumiana Dimova, and Reinhard Lipowsky. Controlled division of cell-sized vesicles by low densities of membrane-bound proteins. *Nature Communications*, 11(1), 2020. ISSN 20411723. doi: 10.1038/s41467-020-14696-0.

[15] Ting F. Zhu and Jack W. Szostak. Coupled growth and division of model protocell membranes. *Journal of the American Chemical Society*, 131(15), 2009. ISSN 00027863. doi: 10.1021/ja900919c.

[16] Yannik Dreher, Kevin Jahnke, Elizaveta Bobkova, Joachim P. Spatz, and Kerstin Göpfrich. Division and Regrowth of Phase-Separated Giant Unilamellar Vesicles**. *Angewandte Chemie - International Edition*, 60(19), 2021. ISSN 15213773. doi: 10.1002/anie.202014174.

[17] Felix Frey and Timon Idema. More than just a barrier: using physical models to couple membrane shape to cell function. *Soft Matter*, 2021. ISSN 1744-683X. doi: 10.1039/d0sm01758b.

[18] Maximilian Fürthauer and Marcos González-Gaitán. Endocytosis and mitosis: A two-way relationship. *Cell Cycle*, 8(20), 2009. ISSN 15514005. doi: 10.4161/cc.8.20.9700.

[19] Charlotte Nugues, Nordine Helassa, Dayani Rajamanoharan, Robert D. Burgoyne, and Lee P. Haynes. Lysosome exocytosis is required for mitosis. *bioRxiv*, 2018. doi: 10.1101/375816.

[20] Willem Kasper Spoelstra, Siddharth Deshpande, and Cees Dekker. Tailoring the appearance: what will synthetic cells look like? *Current Opinion in Biotechnology*, 51, 2018. ISSN 18790429. doi: 10.1016/j.copbio.2017.11.005.

[21] Peter Walde, Katia Cosentino, Helen Engel, and Pasquale Stano. Giant Vesicles: Preparations and Applications. *ChemBioChem*, 2010. ISSN 14394227. doi: 10.1002/cbic.201000010.

[22] Wayne M. Becker, Lewis J. Kleinsmith, Jeff Hardin, and Gregory Paul Bertoni. *The World of the Cell, 7th Edition*. Benjamin Cummings, San Francisco, 2009.

[23] Jiafang Piao, Wei Yuan, and Yuanchen Dong. Recent Progress of DNA Nanostructures on Amphiphilic Membranes. *Macromolecular Bioscience*, 21(5), 2021. ISSN 16165195. doi: 10.1002/mabi.202000440.

[24] John P. Reeves and Robert M. Dowben. Formation and properties of thin-walled phospholipid vesicles. *Journal of Cellular Physiology*, 73(1), 1969. ISSN 10974652. doi: 10.1002/jcp.1040730108.

[25] Pier Luigi Luisi and Peter Walde. *Giant Vesicles: Perspectives in Supramolecular Chemistry*, volume 6. 2007. doi: 10.1002/9780470511534.

[26] Andreas Weinberger, Feng Ching Tsai, Gijsje H. Koenderink, Thais F. Schmidt, Rosângela Itri, Wolfgang Meier, Tatiana Schmatko, André Schröder, and Carlos Marques. Gel-assisted formation of giant unilamellar vesicles. *Biophysical Journal*, 105(1), 2013. ISSN 00063495. doi: 10.1016/j.bpj.2013.05.024.

[27] Sophie Pautot, Barbara J. Frisken, and D. A. Weitz. Production of unilamellar vesicles using an inverted emulsion. *Langmuir*, 19(7), 2003. ISSN 07437463. doi: 10.1021/la026100v.

[28] Manouk Abkarian, Etienne Loiseau, and Gladys Massiera. Continuous droplet interface crossing encapsulation (cDICE) for high throughput monodisperse vesicle design. *Soft Matter*, 7(10), 2011. ISSN 1744683X. doi: 10.1039/c1sm05239j.

[29] Iris Lambert. Synthetic cell division machinery: Towards a dynamic, formin nucleated actin cortex in liposomes. Master's thesis, Delft, 6 2021.

[30] Siddharth Deshpande, Sreekar Wunnava, David Hueting, and Cees Dekker. Membrane Tension–Mediated Growth of Liposomes. *Small*, 2019. ISSN 16136829. doi: 10.1002/smll.201902898.

[31] Lori Van De Cauter, Federico Fanalista, Lennard Van Buren, Nicola De Franceschi, Elisa Godino, Sharon Bouw, Christophe Danelon, Cees Dekker, Gijsje H. Koenderink, and Kristina A. Ganzinger. Optimized cDICE for Efficient Reconstitution of Biological Systems in Giant Unilamellar Vesicles. *ACS Synthetic Biology*, 10(7), 2021. ISSN 21615063. doi: 10.1021/acssynbio.1c00068.

[32] Hannah Stein, Susann Spindler, Navid Bonakdar, Chun Wang, and Vahid Sandoghdar. Production of isolated giant unilamellar vesicles under high salt concentrations. *Frontiers in Physiology*, 8(FEB), 2017. ISSN 1664042X. doi: 10.3389/fphys.2017.00063.

[33] B. Z. Lin, C. C. Yin, and H. Hauser. The effect of positive and negative pH-gradients on the stability of small unilamellar vesicles of negatively charged phospholipids. *BBA - Biomembranes*, 1993. ISSN 00052736. doi: 10.1016/0005-2736(93)90008-N.

[34] Duco Blanken, David Foschepoth, Adriana Calaça Serrão, and Christophe Danelon. Genetically controlled membrane synthesis in liposomes. *Nature Communications*, 11(1), 2020. ISSN 20411723. doi: 10.1038/s41467-020-17863-5.

[35] Leonid V. Chernomordik and Michael M. Kozlov. Mechanics of membrane fusion. *Nature Structural and Molecular Biology*, 15(7), 2008. ISSN 15459993. doi: 10.1038/nsmb.1455.

[36] Lin Yang and Huey W. Huang. Observation of a membrane fusion intermediate structure. *Science*, 297(5588), 2002. ISSN 00368075. doi: 10.1126/science.1074354.

[37] Jörg Nikolaus, Martin Stöckl, Dieter Langosch, Rudolf Volkmer, and Andreas Herrmann. Direct visualization of large and protein-free hemifusion diaphragms. *Biophysical Journal*, 98(7), 2010. ISSN 15420086. doi: 10.1016/j.bpj.2009.11.042.

[38] Avishay Efrat, Leonid V. Chernomordik, and Michael M. Kozlov. Point-like protrusion as a prestalk intermediate in membrane fusion pathway. *Biophysical Journal*, 92(8), 2007. ISSN 00063495. doi: 10.1529/biophysj.106.103341.

[39] Leonid V. Chernomordik and Michael M. Kozlov. Protein-lipid interplay in fusion and fission of biological membranes. *Annual Review of Biochemistry*, 72, 2003. ISSN 00664154. doi: 10.1146/annurev.biochem.72.121801.161504.

[40] Peter M. Kasson and Vijay S. Pande. Control of membrane fusion mechanism by lipid composition: Predictions from ensemble molecular dynamics. *PLoS Computational Biology*, 3(11), 2007. ISSN 1553734X. doi: 10.1371/journal.pcbi.0030220.

[41] Sergey A. Akimov, Rodion J. Molotkovsky, Peter I. Kuzmin, Timur R. Galimzyanov, and Oleg V. Batishchev. Continuum models of membrane fusion: Evolution of the theory. *International Journal of Molecular Sciences*, 21(11), 2020. ISSN 14220067. doi: 10.3390/ijms21113875.

[42] Vladimir S. Malinin, Peter Frederik, and Barry R. Lentz. Osmotic and curvature stress affect PEG-induced fusion of lipid vesicles but not mixing of their lipids. *Biophysical Journal*, 82(4), 2002. ISSN 00063495. doi: 10.1016/S0006-3495(02)75556-2.

[43] Olivier Biner, Thomas Schick, Yannic Müller, and Christoph von Ballmoos. Delivery of membrane proteins into small and giant unilamellar vesicles by charge-mediated fusion. *FEBS Letters*, 2016. ISSN 18733468. doi: 10.1002/1873-3468.12233.

[44] Michael M. Kozlov and Leonid V. Chernomordik. Membrane tension and membrane fusion. *Current Opinion in Structural Biology*, 33, 2015. ISSN 1879033X. doi: 10.1016/j.sbi.2015.07.010.

[45] Fredric S. Cohen, Joshua Zimmerberg, and Alan Finkelstein. Fusion of phospholipid vesicles with planar phospholipid bilayer membranes: II. Incorporation of a vesicular membrane marker into the planar membrane. *Journal of General Physiology*, 75(3), 1980. ISSN 15407748. doi: 10.1085/jgp.75.3.251.

[46] Gerard Apodaca. Modulation of membrane traffic by mechanical stimuli. *American Journal of Physiology - Renal Physiology*, 282(2 51-2), 2002. ISSN 03636127. doi: 10.1152/ajprenal.2002.282.2.f179.

[47] Michael P. Sheetz. Cell control by membrane-cytoskeleton adhesion. *Nature Reviews Molecular Cell Biology*, 2(5), 2001. ISSN 14710072. doi: 10.1038/35073095.

[48] Marcos Arribas Perez and Paul A. Beales. Biomimetic Curvature and Tension-Driven Membrane Fusion Induced by Silica Nanoparticles. *Langmuir*, 37(47), 2021. ISSN 15205827. doi: 10.1021/acs.langmuir.1c02492.

[49] Colin P.S. Tilcock and Derek Fisher. Interaction of phospholipid membranes with poly(ethylene glycol)s. *BBA - Biomembranes*, 557(1), 1979. ISSN 00052736. doi: 10.1016/0005-2736(79)90089-0.

[50] L. T. Boni, T. P. Stewart, J. L. Alderfer, and S. W. Hui. Lipid-polyethylene glycol interactions: II. Formation of defects in bilayers. *The Journal of Membrane Biology*, 62(1-2), 1981. ISSN 00222631. doi: 10.1007/BF01870201.

[51] Timothy J. Aldwinckle, Quet F. Ahkong, Alec D. Bangham, Derek Fisher, and Jack A. Lucy. Effects of poly(ethylene glycol) on liposomes and erythrocytes. Permeability changes and membrane fusion. *BBA - Biomembranes*, 689(3), 1982. ISSN 00052736. doi: 10.1016/0005-2736(82)90313-3.

[52] Rafael B. Lira, Tom Robinson, Rumiana Dimova, and Karin A. Riske. Highly Efficient Protein-free Membrane Fusion: A Giant Vesicle Study. *Biophysical Journal*, 2019. ISSN 15420086. doi: 10.1016/j.bpj.2018.11.3128.

[53] Rafael B. Lira, Fernanda S.C. Leomil, Renan J. Melo, Karin A. Riske, and Rumiana Dimova. To close or to collapse: The role of charges on membrane stability upon pore formation. *bioRxiv*, 2020. ISSN 26928205. doi: 10.1101/2020.08.31.274860.

[54] Robert R. Ishmukhametov, Aidan N. Russell, and Richard M. Berry. A modular platform for one-step assembly of multi-component membrane systems by fusion of charged proteoliposomes. *Nature Communications*, 7, 2016. ISSN 20411723. doi: 10.1038/ncomms13025.

[55] Jessica L. Symons, Kwang Jin Cho, Jeffrey T. Chang, Guangwei Du, M. Neal Waxham, John F. Hancock, Ilya Levental, and Kandice R. Levental. Lipidomic atlas of mammalian cell membranes reveals hierarchical variation induced by culture conditions, subcellular membranes, and cell lineages. *Soft Matter*, 17(2), 2021. ISSN 17446848. doi: 10.1039/d0sm00404a.

[56] Weiming Xu, Jing Wang, James E. Rothman, and Frédéric Pincet. Accelerating SNARE-Mediated Membrane Fusion by DNA-Lipid Tethers. *Angewandte Chemie - International Edition*, 54(48), 2015. ISSN 15213773. doi: 10.1002/anie.201506844.

[57] R Jahn, T Lang, and Tc Südhof. Jahn, R., Lang, T., & Südhof, T. (2003). Membrane fusion. Cell, 112(4), 519–533. http://doi.org/10.1371/journal.pone.0007375.oMembrane fusion. *Cell*, 112(4), 2003.

[58] Thomas Weber, Boris V. Zemelman, James A. McNew, Benedikt Westermann, Michael Gmachl, Francesco Parlati, Thomas H. Söllner, and James E. Rothman. SNAREpins: Minimal machinery for membrane fusion. *Cell*, 92(6), 1998. ISSN 00928674. doi: 10.1016/S0092-8674(00)81404-X.

[59] Gudrun Stengel, Raphael Zahn, and Fredrik Höök. DNA-induced programmable fusion of phospholipid vesicles. *Journal of the American Chemical Society*, 129(31), 2007. ISSN 00027863. doi: 10.1021/ja073200k.

[60] S.E. Lychevski. *Nano and Molecular Electronics Handbook*. CRC Press, 2007. doi: 10.1201/9781315221670.

[61] Gudrun Stengel, Lisa Simonsson, Richard A. Campbell, and Fredrik Höök. Determinants for membrane fusion induced by cholesterol-modified DNA zippers. *Journal of Physical Chemistry B*, 112(28), 2008. ISSN 15206106. doi: 10.1021/jp802005b.

[62] Zhuojun Meng, Jian Yang, Qing Liu, Jan Willem de Vries, Agnieszka Gruszka, Alberto Rodríguez-Pulido, Bart J. Crielaard, Alexander Kros, and Andreas Herrmann. Efficient Fusion of Liposomes by Nucleobase Quadruple-Anchored DNA. *Chemistry - A European Journal*, 23 (39), 2017. ISSN 15213765. doi: 10.1002/chem.201701379.

[63] Kristina M. Flavier and Steven G. Boxer. Vesicle Fusion Mediated by Solanesol-Anchored DNA. *Biophysical Journal*, 113(6), 2017. ISSN 15420086. doi: 10.1016/j.bpj.2017.05.034.

[64] Minsub Chung, Randall D. Lowe, Yee Hung M. Chan, Prasad V. Ganesan, and Steven G. Boxer. DNA-tethered membranes formed by giant vesicle rupture. *Journal of Structural Biology*, 168 (1), 2009. ISSN 10478477. doi: 10.1016/j.jsb.2009.06.015.

[65] Yee-Hung M. Chan, Bettina van Lengerich, and Steven G. Boxer. Lipid-anchored DNA mediates vesicle fusion as observed by lipid and content mixing. *Biointerphases*, 3(2), 2008. ISSN 1934-8630. doi: 10.1116/1.2889062.

[66] Yee Hung M. Chan, Bettina Van Lengerich, and Steven G. Boxer. Effects of linker sequences on vesicle fusion mediated by lipid-anchored DNA oligonucleotides. *Proceedings of the National Academy of Sciences of the United States of America*, 106(4), 2009. ISSN 00278424. doi: 10.1073/pnas.0812356106.

[67] Lisa Simonsson, Peter Jönsson, Gudrun Stengel, and Fredrik Höök. Site-specific DNA-controlled fusion of single lipid vesicles to supported lipid bilayers. *ChemPhysChem*, 11(5), 2010. ISSN 14397641. doi: 10.1002/cphc.200901010.

[68] Jan Wilschut, Nejat Düzgüneş, Robert Fraley, and Demetrios Papahadjopoulos. Studies on the Mechanism of Membrane Fusion: Kinetics of Calcium Ion Induced Fusion of Phosphatidylserine Vesicles Followed by a New Assay for Mixing of Aqueous Vesicle Contents. *Biochemistry*, 19 (26), 1980. ISSN 15204995. doi: 10.1021/bi00567a011.

[69] Bettina Van Lengerich, Robert J. Rawle, Poul Martin Bendix, and Steven G. Boxer. Individual vesicle fusion events mediated by lipid-anchored DNA. *Biophysical Journal*, 105(2), 2013. ISSN 00063495. doi: 10.1016/j.bpj.2013.05.056.

[70] Jakob Andersson and Ingo Köper. Tethered and polymer supported bilayer lipid membranes: Structure and function. *Membranes*, 6(2), 2016. ISSN 20770375. doi: 10.3390/membranes6020030.

[71] Benjamin Kollmitzer, Peter Heftberger, Michael Rappolt, and Georg Pabst. Monolayer spontaneous curvature of raft-forming membrane lipids. *Soft Matter*, 9(45), 2013. ISSN 17446848. doi: 10.1039/c3sm51829a.

[72] Justin A. Peruzzi, Miranda L. Jacobs, Timothy Q. Vu, Kenneth S. Wang, and Neha P. Kamat. Barcoding Biological Reactions with DNA-Functionalized Vesicles. *Angewandte Chemie - International Edition*, 58(51), 2019. ISSN 15213773. doi: 10.1002/anie.201911544.

[73] Diana Morzy, Roger Rubio-Sánchez, Himanshu Joshi, Aleksei Aksimentiev, Lorenzo Di Michele, and Ulrich F. Keyser. Cations Regulate Membrane Attachment and Functionality of DNA Nanostructures. *Journal of the American Chemical Society*, 143(19), 2021. ISSN 15205126. doi: 10.1021/jacs.1c00166.

[74] Lodish Harvey, Berk Arnold, Zipursky S Lawrence, l Matsudaira Pau, Baltimore David, and Darnel James. *Molecular Cell Biology. 4th edition*. 2000. doi: 10.1016/j.jasms.2009.08.001.

[75] K.R. Spring. Introduction to Fluorescence Microscopy, 2022. URL https://www.microscopyu.com/techniques/fluorescence/introduction-to-fluorescence-microscopy. Date accessed: 02-02-2022.

[76] ONI. Epifluorescence microscopy, 2022. URL https://oni.bio/nanoimager/super-resolution-microscopy/epifluorescence-microscopy/#:~:text=What%20is%20epifluorescence%20microscopy%3F,intensity%20light%20source%20is%20used. Date accessed: 02-02-2022.

[77] Max Born, Emil Wolf, and Eugene Hecht. Principles of Optics: Electromagnetic Theory of Propagation, Interference and Diffraction of Light . *Physics Today*, 53(10), 2000. ISSN 0031-9228. doi: 10.1063/1.1325200.

[78] AAT Bioquest. What is fluorescence crosstalk?, 11 2019. URL https://www.aatbio.com/resources/faq-frequently-asked-questions/What-is-fluorescence-crosstalk. Date accessed: 02-02-2022.

[79] Rafael B. Lira and Rumiana Dimova. Fusion assays for model membranes: a critical review. *Advances in Biomembranes and Lipid Self-Assembly*, 30, 2019. ISSN 24519634. doi: 10.1016/bs.abl.2019.09.003.

[80] Philipp M.G. Löffler, Oliver Ries, Alexander Rabe, Anders H. Okholm, Rasmus P. Thomsen, Jørgen Kjems, and Stefan Vogel. A DNA-Programmed Liposome Fusion Cascade. *Angewandte Chemie - International Edition*, 56(43), 2017. ISSN 15213773. doi: 10.1002/anie.201703243.

[81] Haijia Yu, Shailendra S. Rathore, Jamie A. Lopez, Eric M. Davis, David E. James, Jennifer L. Martin, and Jingshi Shen. Comparative studies of Munc18c and Munc18-1 reveal conserved and divergent mechanisms of Sec1/Munc18 proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 110(35), 2013. ISSN 00278424. doi: 10.1073/pnas.1311232110.

[82] Nestor Lopez Mora, Aimee L. Boyle, Bart Jan van Kolck, Anouk Rossen, Šárka Pokorná, Alena Koukalová, Radek Šachl, Martin Hof, and Alexander Kros. Controlled Peptide-Mediated Vesicle Fusion Assessed by Simultaneous Dual-Colour Time-Lapsed Fluorescence Microscopy. *Scientific Reports*, 10(1), 2020. ISSN 20452322. doi: 10.1038/s41598-020-59926-z.

[83] Mukarram A. Tahir, Zekiye P. Guven, Laura R. Arriaga, Berta Tinao, Yu Sang Sabrina Yang, Ahmet Bekdemir, Jacob T. Martin, Alisha N. Bhanji, Darrell Irvine, Francesco Stellacci, and Alfredo Alexander-Katz. Calcium-triggered fusion of lipid membranes is enabled by amphiphilic nanoparticles. *Proceedings of the National Academy of Sciences of the United States of America*, 117 (31), 2020. ISSN 10916490. doi: 10.1073/pnas.1902597117.

[84] Ran Tivony, Marcus Fletcher, Kareem Al Nahas, and Ulrich F. Keyser. A Microfluidic Platform for Sequential Assembly and Separation of Synthetic Cell Models. *ACS Synthetic Biology*, 10(11), 2021. ISSN 21615063. doi: 10.1021/acssynbio.1c00371.

[85] Lennard Van Buren. *Synthetic Cell Aspirations*. PhD thesis, Delft University of Technology, Delft, 2022.

[86] Wooli Bae, Tae Young Yoon, and Cherlhyun Jeong. Direct evaluation of self-quenching behavior of fluorophores at high concentrations using an evanescent field. *PLoS ONE*, 16(2 February 2021), 2021. ISSN 19326203. doi: 10.1371/journal.pone.0247326.

[87] Lennard Van Buren, Gijsje Hendrika Koenderink, and Cristina Martinez-Tores. DisGUVery: a versatile open-source software for high-throughput image analysis of Giant Unilamellar Vesicles. *BioRxiv*, 1 2022.

[88] HK Yuen, J. Princen, J. Illingworth, and J. Kittler. Comparative study of Hough Transform methods for circle finding. *Image and Vision Computing*, 8(1), 1990. ISSN 02628856. doi: 10.1016/0262-8856(90)90059-E.

[89] Sourav Bhattacharjee. DLS and zeta potential - What they are and what they are not? *Journal of Controlled Release*, 235, 2016. ISSN 18734995. doi: 10.1016/j.jconrel.2016.06.017.

[90] Ulf Nobbmann (Malvern Panalytical). Intensity-Volume-Number: Which size is correct?, 1 2017. URL `https://www.materials-talks.com/intensity-volume-number-which-size-is-correct/`. Date accessed: 03-02-2022.

[91] T. G.F. Souza, V. S.T. Ciminelli, and N. D.S. Mohallem. A comparison of TEM and DLS methods to characterize size distribution of ceramic nanoparticles. In *Journal of Physics: Conference Series*, volume 733, 2016. doi: 10.1088/1742-6596/733/1/012039.

[92] Steven Shimizu. fitAdsorptionIsotherm, MATLAB Central File Exchange, 2021. URL `https://nl.mathworks.com/matlabcentral/fileexchange/46981-fitadsorptionisotherm`. Date accessed: 28-12-2021.

[93] Irving Langmuir. The adsorption of gases on plane surfaces of glass, mica and platinum. *Journal of the American Chemical Society*, 40(9), 1918. ISSN 15205126. doi: 10.1021/ja02242a004.

[94] A. Mora-Boza, T. Lopes-Costa, F. Gámez, and J. M. Pedrosa. Unveiling the interaction of DNA-octadecylamine at the air-water interface by ultraviolet-visible reflection spectroscopy. *RSC Advances*, 7(10), 2017. ISSN 20462069. doi: 10.1039/c6ra27903a.

[95] Indriati Pfeiffer and Fredrik Höök. Bivalent cholesterol-based coupling of oligonucletides to lipid membrane assemblies. *Journal of the American Chemical Society*, 126(33), 2004. ISSN 00027863. doi: 10.1021/ja048514b.

[96] Stef A.J. Van Der Meulen, Galina V. Dubacheva, Marileen Dogterom, Ralf P. Richter, and Mirjam E. Leunissen. Quartz crystal microbalance with dissipation monitoring and spectroscopic ellipsometry measurements of the phospholipid bilayer anchoring stability and kinetics of hydrophobically modified DNA oligonucleotides. *Langmuir*, 30(22), 2014. ISSN 15205827. doi: 10.1021/la500940a.

[97] Integrated DNA Technologies. OligoAnalyzer, 2022. URL `https://eu.idtdna.com/calc/analyzer`. Date accessed: 11-01-2022.

[98] Minjoung Kyoung, Ankita Srivastava, Yunxiang Zhang, Jiajie Diao, Marija Vrljic, Patricia Grob, Eva Nogales, Steven Chu, and Axel T. Brunger. In vitro system capable of differentiating fast Ca 2+-triggered content mixing from lipid exchange for mechanistic studies of neurotransmitter release. *Proceedings of the National Academy of Sciences of the United States of America*, 108 (29), 2011. ISSN 00278424. doi: 10.1073/pnas.1107900108.

[99] Dawn Ronan, Nathalie Sordé, and Stefan Matile. Blocker efflux through blocked pores. In *Journal of Physical Organic Chemistry*, volume 17, 2004. doi: 10.1002/poc.818.

[100] Susanne Kreye, Jörg Malsam, and Thomas H. Söllner. In vitro assays to measure SNARE-Mediated vesicle fusion. *Methods in Molecular Biology*, 440, 2008. ISSN 10643745. doi: 10.1007/978-1-59745-178-9{_}3.

[101] Hanna Barman, Michael Walch, Sonja Latinovic-Golic, Claudia Dumrese, Max Dolder, Peter Groscurth, and Urs Ziegler. Cholesterol in negatively charged lipid bilayers modulates the effect of the antimicrobial protein granulysin. *Journal of Membrane Biology*, 212(1), 2006. ISSN 00222631. doi: 10.1007/s00232-006-0040-3.

[102] F. R. Hallett, J. Marsh, B. G. Nickel, and J. M. Wood. Mechanical properties of vesicles. II. A model for osmotic swelling and lysis. *Biophysical Journal*, 64(2), 1993. ISSN 00063495. doi: 10.1016/S0006-3495(93)81384-5.

[103] Karina Alleva, Osvaldo Chara, and Gabriela Amodeo. Aquaporins: Another piece in the osmotic puzzle. *FEBS Letters*, 586(19), 2012. ISSN 00145793. doi: 10.1016/j.febslet.2012.06.013.

[104] Adamala Lab. Liposome calculation, 3 2021. URL `http://protobiology.org/wp3/2021/03/17/liposomecalculation/`. Date accessed: 05-02-2022.

[105] A. Sonnleitner, G. J. Schütz, and Thomas Schmidt. Free Brownian motion of individual lipid molecules in biomembranes. *Biophysical Journal*, 77(5), 1999. ISSN 00063495. doi: 10.1016/S0006-3495(99)77097-9.

[106] Magdalena Przybylo, Jan Sýkora, Jana Humpolíčová, Aleš Benda, Anna Zan, and Martin Hof. Lipid diffusion in giant unilamellar vesicles is more than 2 times faster than in supported phospholipid bilayers under identical conditions. *Langmuir*, 22(22), 2006. ISSN 07437463. doi: 10.1021/la061934p.

[107] Chen Ming Chang, Yuk Gyn Lau, Shu Ching Ou, Tse Yu Lin, and Wen Tau Juan. Anomalous diffusion of DNA on a supported cationic lipid membrane. *EPL*, 109(3), 2015. ISSN 12864854. doi: 10.1209/0295-5075/109/38002.

[108] Christopher Miller, Peter Arvan, John N. Telford, and Efraim Racker. Ca++-induced fusion of proteoliposomes: Dependence on transmembrane osmotic gradient. *The Journal of Membrane Biology*, 30(1), 1976. ISSN 00222631. doi: 10.1007/BF01869672.

[109] Karin A. Riske and Rumiana Dimova. Electro-deformation and poration of giant vesicles viewed with high temporal resolution. *Biophysical Journal*, 2005. ISSN 00063495. doi: 10.1529/biophysj.104.050310.

[110] Miao Yu, Rafael B. Lira, Karin A. Riske, Rumiana Dimova, and Hao Lin. Ellipsoidal Relaxation of Deformed Vesicles. *Physical Review Letters*, 115(12), 2015. ISSN 10797114. doi: 10.1103/PhysRevLett.115.128303.

[111] Jérôme Solon, Jacques Pécréaux, Philippe Girard, Marie Claude Fauré, Jacques Prost, and Patricia Bassereau. Negative tension induced by lipid uptake. *Physical Review Letters*, 97(9), 2006. ISSN 00319007. doi: 10.1103/PhysRevLett.97.098103.

[112] Adai Colom, Emmanuel Derivery, Saeideh Soleimanpour, Caterina Tomba, Marta Dal Molin, Naomi Sakai, Marcos González-Gaitán, Stefan Matile, and Aurélien Roux. A fluorescent membrane tension probe. *Nature Chemistry*, 10(11), 2018. ISSN 17554349. doi: 10.1038/s41557-018-0127-3.

[113] Mike May. Focal Wars: Widefield vs. Confocal, 11 2018. URL https://www.biocompare.com/Editorial-Articles/355647-Focal-Wars-Widefield-vs-Confocal/#:~:text=In%20a%20widefield%20microscope%2C%20the,one%20beam%20in%20some%20platforms. Date accessed: 07-02-2022.

[114] The Synthetic Cell initiative, 2021. URL https://www.syntheticcell.eu/. Date accessed: 09-07-2021.

[115] The French Alternative Energies and Atomic Energy Commission (CEA). The CEA: a key player in technological research, 2021. URL https://www.cea.fr/english/Pages/cea/the-cea-a-key-player-in-technological-research.aspx. Date accessed: 12-07-2021.

[116] The Kavli Foundation. About - Advancing science for the benefit of humanity., 2021. URL https://kavlifoundation.org/about. Date accessed: 12-07-2021.

[117] Leonardo Augusto de Vasconcelos Gomes, Ana Lucia Figueiredo Facin, Mario Sergio Salerno, and Rodrigo Kazuo Ikenami. Unpacking the innovation ecosystem construct: Evolution, gaps and trends. *Technological Forecasting and Social Change*, 136, 2018. ISSN 00401625. doi: 10.1016/j.techfore.2016.11.009.

[118] L Ikpaahindi. An overview of bibliometrics: its measurements, laws and their applications. *Libri (Copenhagen)*, 35(2), 1985. ISSN 0024-2667.

[119] Deog Seong Oh, Fred Phillips, Sehee Park, and Eunghyun Lee. Innovation ecosystems: A critical examination. In *Technovation*, volume 54, 2016. doi: 10.1016/j.technovation.2016.02.004.

[120] Ove Granstrand and Marcus Holgersson. Innovation ecosystems: A conceptual review and a new definition. *Technovation*, 90-91, 2020. ISSN 01664972. doi: 10.1016/j.technovation.2019.102098.

[121] Ron Adner and Rahul Kapoor. Value creation in innovation ecosystems: How the structure of technological interdependence affects firm performance in new technology generations. *Strategic Management Journal*, 31(3), 2010. ISSN 01432095. doi: 10.1002/smj.821.

[122] Benoît Godin. *Innovation contested: The idea of innovation over the centuries*. 2015. doi: 10.4324/9781315855608.

[123] Duncan R. Shaw and Tim Allen. Studying innovation ecosystems using ecology theory. *Technological Forecasting and Social Change*, 136, 2018. ISSN 00401625. doi: 10.1016/j.techfore.2016.11.030.

[124] J. F. Moore. Predators and prey: a new ecology of competition. *Harvard Business Review*, 71 (3), 1993. ISSN 00178012.

[125] Joseph A. Schumpeter. *Capitalism, Socialism, and Democracy*. Harper and Row, New York, NY, 1942.

[126] Ondřej Žižlavský. Past, present and future of the innovation process. *International Journal of Engineering Business Management*, 5(1), 2013. ISSN 18479790. doi: 10.5772/56920.

[127] Roy Rothwell. Towards the Fifth-generation Innovation Process. *International Marketing Review*, 11(1), 1994. ISSN 02651335. doi: 10.1108/02651339410057491.

[128] Elias G. Carayannis and David F.J. Campbell. 'Mode 3' and 'Quadruple Helix': Toward a 21st century fractal innovation ecosystem. *International Journal of Technology Management*, 46(3-4), 2009. ISSN 02675730. doi: 10.1504/ijtm.2009.023374.

[129] Michael Gibbons, Camille Limoges, Helga Nowotny, Simon Schwartzman, Peter Scott, and Martin Trow. The new production of knowledge. *Social Studies Of Science*, 32(4), 1994. ISSN 03063127.

[130] Henry Etzkowitz, Andrew Webster, Christiane Gebhardt, and Branca Regina Cantisano Terra. The future of the university and the university of the future: Evolution of ivory tower to entrepreneurial paradigm. *Research Policy*, 2000. ISSN 00487333. doi: 10.1016/S0048-7333(99)00069-4.

[131] Sheila Jasanoff. *Designs on nature: Science and democracy in Europe and the United States*. Princeton University Press, Princeton, NJ, 2007.

[132] Mario Pansera, Richard Owen, Darian Meacham, and Vivienne Kuh. Embedding responsible innovation within synthetic biology research and innovation: insights from a UK multi-disciplinary research centre. *Journal of Responsible Innovation*, 7(3):384–409, 2020. ISSN 23299037. doi: 10.1080/23299460.2020.1785678.

[133] Andrew S. Balmer, Jane Calvert, Claire Marris, Susan Molyneux-Hodgson, Emma Frow, Matthew Kearnes, Kate Bulpin, Pablo Schyfter, Adrian MacKenzie, and Paul Martin. Taking roles in interdisciplinary collaborations: Reflections on working in post-ELSI spaces in the UK synthetic biology community. *Science and Technology Studies*, 28(3), 2015. ISSN 22434690. doi: 10.23987/sts.55340.

[134] Richard Owen, Phil Macnaghten, and Jack Stilgoe. Responsible research and innovation: From science in society to science for society, with society. *Science and Public Policy*, 2012. ISSN 03023427. doi: 10.1093/scipol/scs093.

[135] Richard Owen and Mario Pansera. Responsible Innovation and Responsible Research and Innovation. In *Handbook on Science and Public Policy*, chapter 2, pages 26–48. Edward Elgar publishing, 2019.

[136] Jack Stilgoe, Richard Owen, and Phil Macnaghten. Developing a framework for responsible innovation. *Research Policy*, 42(9), 2013. ISSN 00487333. doi: 10.1016/j.respol.2013.05.008.

[137] Zenlin Kwee, Emad Yaghmaei, and Steven Flipse. Responsible research and innovation in practice an exploratory assessment of Key Performance Indicators (KPIs) in a Nanomedicine Project. *Journal of Responsible Technology*, 5(September 2016):100008, 2021. ISSN 26666596. doi: 10.1016/j.jrt.2021.100008.

[138] Ibo van de Poel, Lotte Asveld, Steven Flipse, Pim Klaassen, Victor Scholten, and Emad Yaghmaei. Company strategies for responsible research and innovation (RRI): A conceptual model. *Sustainability (Switzerland)*, 9(11), 2017. ISSN 20711050. doi: 10.3390/su9112045.

[139] Hannah Monsrud Sandvik. On Responsible Research and Innovation (and what we do when we do things with words), 12 2018. URL `https://www.fpol.no/on-responsible-research-and-innovation-and-what-we-do-when-we-do-things-with-words/`. Date accessed: 03-03-2022.

[140] RRI-Practice. Responsible Research and Innovation in Practice, 2016. URL `https://www.rri-practice.eu/`. Date accessed: 06-03-2021.

[141] Clare Shelley-Egan, Mads Dahl Gjefsen, and Rune Nydal. Consolidating RRI and Open Science: Understanding the potential for transformative change. *Life Sciences, Society and Policy*, 16(1), 2020. ISSN 21957819. doi: 10.1186/s40504-020-00103-5.

[142] Ibo van de Poel, Lotte Asveld, Steven Flipse, Pim Klaassen, Zenlin Kwee, Maria Maia, Elvio Mantovani, Christopher Nathan, Andrea Porcari, and Emad Yaghmaei. Learning to do responsible innovation in industry: six lessons. *Journal of Responsible Innovation*, 2020. ISSN 23299037. doi: 10.1080/23299460.2020.1791506.

[143] Carol Mershon and Olga Shvetsova. *Formal Modeling in Social Science*. 2019. doi: 10.3998/mpub.8811571.

[144] Wreally. Transcribe Audio to Text, Fast & Securely, 2021. URL `https://transcribe.wreally.com/`. Date accessed: 15-01-2022.

[145] R.K. Yin. Case study methodology R.K. Yin (2003, 3rd edition). Case Study Research design and methods. Sage, Thousand Oaks (CA)..pdf. In *Case Study Research: design and methods*. 2003.

[146] Johnny Saldaña. The Coding Manual for Qualitative Researchers (No. 14). *Sage*, 2016. ISSN 01631829.

[147] Kathleen M. MacQueen, Eleanor McLellan, Kelly Kay, and Bobby Milstein. Codebook development for team-based qualitative analysis. *Field Methods*, 10(2), 1998. ISSN 1525822X. doi: 10.1177/1525822X980100020301.

[148] Scopus. Content Coverage Guide. Technical report, 2020. URL `https://www.elsevier.com/__data/assets/pdf_file/0007/69451/Scopus_ContentCoverage_Guide_WEB.pdf`. Date accessed: 12-01-2022.

[149] Giovanni Schiuma and Daniela Carlucci. Managing strategic partnerships with universities in innovation ecosystems: A research agenda. *Journal of Open Innovation: Technology, Market, and Complexity*, 4(3), 2018. ISSN 21998531. doi: 10.3390/joitmc4030025.

[150] Lei Ma, Zheng Liu, Xiaojing Huang, and Tao Li. The Impact of Local Government Policy on Innovation Ecosystem in Knowledge Resource Scarce Region: Case Study of Changzhou, China. *Science, Technology and Society*, 24(1), 2019. ISSN 09730796. doi: 10.1177/0971721818806096.

[151] Guannan Xu, Yuchen Wu, Tim Minshall, and Yuan Zhou. Exploring innovation ecosystems across science, technology, and business: A case of 3D printing in China. *Technological Forecasting and Social Change*, 136, 2018. ISSN 00401625. doi: 10.1016/j.techfore.2017.06.030.

[152] Olga A. Shvetsova and Sang Kon Lee. Living labs in university-industry cooperation as a part of innovation ecosystem: Case study of south korea. *Sustainability (Switzerland)*, 13(11), 2021. ISSN 20711050. doi: 10.3390/su13115793.

[153] Yu Shan Su, Zong Xi Zheng, and Jin Chen. A multi-platform collaboration innovation ecosystem: the case of China. *Management Decision*, 56(1), 2018. ISSN 00251747. doi: 10.1108/MD-04-2017-0386.

[154] Minoru Hayashida, Tomohiro Anzai, and Hiromichi Kimura. Establishing of a base to build "the innovation ecosystem". *Drug Delivery System*, 30(3), 2015. ISSN 18812732. doi: 10.2745/dds.30.184.

[155] Zheng Liu and Victoria Stephens. Exploring innovation ecosystem from the perspective of sustainability: Towards a conceptual framework. *Journal of Open Innovation: Technology, Market, and Complexity*, 5(3), 2019. ISSN 21998531. doi: 10.3390/joitmc5030048.

[156] John Butler and David Gibson. Research universities in the framework of regional innovation ecosystem: The case of Austin, Texas. *Foresight Russia*, 7(2), 2013. ISSN 1995459X.

[157] Leonie M. Levrouw, Zwanet van Lubek, and Frido Smulders. Suspense as a driver for university-industry collaboration. *Journal of Higher Education Theory and Practice*, 20(13), 2020. ISSN 21583595. doi: 10.33423/jhetp.v20i13.3835.

[158] Admore Tutsirayi Nyamaka, Adele Botha, Judy Van Biljon, and Mario Alphonso Marais. The components of an innovation ecosystem framework for Botswana's mobile applications. *Electronic Journal of Information Systems in Developing Countries*, 86(6), 2020. ISSN 16814835. doi: 10.1002/isd2.12137.

[159] Patrick J. Silva and Kenneth S. Ramos. Academic medical centers as innovation ecosystems: Evolution of industry partnership models beyond the Bayh–Dole act. *Academic Medicine*, 93(8), 2018. ISSN 1938808X. doi: 10.1097/ACM.0000000000002259.

[160] Yanfeng Jiang and Wenyu Zheng. Coupling mechanism of green building industry innovation ecosystem based on blockchain smart city. *Journal of Cleaner Production*, 307, 2021. ISSN 09596526. doi: 10.1016/j.jclepro.2021.126766.

[161] João Lopes, Luís Farinha, and João J. Ferreira. Regional innovation ecosystems and smart specialization: Opportunities and challenges for regions. *Revista Brasileira de Gestao e Desenvolvimento Regional*, 16(1), 2020. ISSN 1809239X.

[162] Francisco José Sáez-Martínez, Ángela González-Moreno, and Teresa Hogan. The role of university in eco-entrepreneurship: Evidence from the eurobarometer survey on attitudes of european entrepreneurs towards eco-innovation. *Environmental Engineering and Management Journal*, 13(10), 2014. ISSN 18433707. doi: 10.30638/eemj.2014.284.

[163] Helmut Traitler, Heribert J. Watzke, and I. Sam Saguy. Reinventing R&D in an Open Innovation Ecosystem. *Journal of Food Science*, 76(2), 2011. ISSN 00221147. doi: 10.1111/j.1750-3841.2010.01998.x.

[164] Bruno Fischer, Maribel Guerrero, José Guimón, and Paola Rücker Schaeffer. Knowledge transfer for frugal innovation: where do entrepreneurial universities stand? *Journal of Knowledge Management*, 25(2), 2021. ISSN 17587484. doi: 10.1108/JKM-01-2020-0040.

[165] Moira Gunn. Multi-disciplined ecosystem-centric bioentrepreneurship education: Case study – University of San Francisco (USF). *Journal of Commercial Biotechnology*, 26(1), 2021. ISSN 1478565X. doi: 10.5912/jcb973.

[166] Nataliya Kravchenko, Almira Yusupova, and Svetlana Kuznetsova. Research and Business Cooperation: International Practice and Siberian Experience. *Journal of Siberian Federal University. Humanities & Social Sciences*, 2019. ISSN 1997-1370. doi: 10.17516/1997-1370-0414.

[167] Cesar Bandera and Ellen Thomas. The Role of Innovation Ecosystems and Social Capital in Startup Survival. *IEEE Transactions on Engineering Management*, 66(4), 2019. ISSN 15580040. doi: 10.1109/TEM.2018.2859162.

[168] F Z De Carvalho, V T Bersani, L F Maldaner, and J M Piqué. Instituições que promovem o empreendedorismo inovador no brasil: Mapeamento e conexões [Institutions that foster innovative entrepreneurship in brazil: Mapping and connections]. *Praksis*, 17(2), 2020. ISSN 24481939.

[169] Diana Joseph, Susan Windham-Bannister, and Mikel Mangold. What corporates can do to help an innovation ecosystem thrive – and why they should do it. *Journal of Commercial Biotechnology*, 26(1), 2021. ISSN 1478565X. doi: 10.5912/jcb975.

[170] Daniel Ferreira Polónia and Adriana Coutinho Gradim. Innovation and knowledge flows in healthcare ecosystems: The portuguese case. *Electronic Journal of Knowledge Management*, 18(3), 2021. ISSN 14794411. doi: 10.34190/EJKM.18.3.2122.

[171] Zheng Liu, Lei Ma, Yue Zhu, and Wenchao Ji. An investigation on responsible innovation in the emerging shared bicycle industry: Case study of a Chinese firm. *Journal of Open Innovation: Technology, Market, and Complexity*, 5(3), 2019. ISSN 21998531. doi: 10.3390/joitmc5030042.

[172] Amon Simba. A new model of knowledge and innovative capability development for small born-global bio-tech firms: Evidence from the east midlands, UK. *International Journal of Entrepreneurship and Innovation Management*, 19(1-2), 2015. ISSN 17415098. doi: 10.1504/IJEIM.2015.068421.

[173] Shilpa and Sujit Bhattacharya. Bilateral S&T organisation as an innovation intermediary: Case study of Indo-French cell for water sciences. *Journal of Scientometric Research*, 9(2 s), 2020. ISSN 23200057. doi: 10.5530/JSCIRES.9.2S.34.

[174] N. Komninos and P. Tsarchopoulos. Toward Intelligent Thessaloniki: From an Agglomeration of Apps to Smart Districts. *Journal of the Knowledge Economy*, 4(2), 2013. ISSN 18687873. doi: 10.1007/s13132-012-0085-8.

[175] Kathrin Cresswell, Robin Williams, Narath Carlile, and Aziz Sheikh. Accelerating innovation in health care: Insights from a qualitative inquiry into United Kingdom and United States innovation centers. *Journal of Medical Internet Research*, 22(9), 2020. ISSN 14388871. doi: 10.2196/19644.

[176] Anna Sinell, Vivien Iffländer, and Antonia Muschner. Uncovering transfer – a cross-national comparative analysis. *European Journal of Innovation Management*, 21(1), 2018. ISSN 14601060. doi: 10.1108/EJIM-01-2017-0006.

[177] BE-Basic. About BE-Basic, 2020. URL `https://be-basic.org/about-be-basic/`. Date accessed: 16-07-2021.

[178] Pablo Schyfter and Jane Calvert. Intentions, Expectations and Institutions: Engineering the Future of Synthetic Biology in the USA and the UK. *Science as Culture*, 24(4), 2015. ISSN 14701189. doi: 10.1080/09505431.2015.1037827.

[179] DNA Script. About us, 2021. URL `https://www.dnascript.com/about-us/`. Date accessed: 10-07-2021.

[180] Synovance, 2021. URL `https://synovance.com/`. Date accessed: 12-07-2021.

[181] Abvance Biotech. About Abvance, 2021. URL `http://www.abvance.com/about-abvance/`. Date accessed: 10-07-2022.

[182] Yealthy. About us, 2021. URL `https://yealthy.life/about.html`. Date accessed: 10-07-2022.

[183] Jim Swartz. Developing cell-free biology for industrial applications. In *Journal of Industrial Microbiology and Biotechnology*, volume 33, 2006. doi: 10.1007/s10295-006-0127-y.

[184] Laura Restrepo-Pérez, Chirlmin Joo, and Cees Dekker. Paving the way to single-molecule protein sequencing. *Nature Nanotechnology*, 13(9), 2018. ISSN 17483395. doi: 10.1038/s41565-018-0236-6.

[185] Oskar Staufer, Jacqueline A De Lora, Eleonora Bailoni, Alisina Bazrafshan, Amelie S Benk, Kevin Jahnke, Zachary A Manzer, Lado Otrin, Telmo Díez Pérez, Judee Sharon, Jan Steinkühler, Katarzyna P Adamala, Bruna Jacobson, Marileen Dogterom, Kerstin Göpfrich, Darko Stefanovic, Susan R Atlas, Michael Grunze, Matthew R Lakin, Andrew P Shreve, Joachim P Spatz, and Gabriel P López. Building a community to engineer synthetic cells and organelles from the bottom-up. *eLife*, 2021. doi: 10.7554/eLife.

[186] Delft University of Technology. Innovatie & Impact, 2022. URL `https://www.tudelft.nl/innovatie-impact`. Date accessed: 09-01-2022.

[187] Delft Enterprises B.V. What we do. 2022. URL `https://www.delftenterprises.nl/`. Date accessed: 13-01-2022.

[188] Biominas Brasil. Technology Readiness Level (TRL), 2022. URL `https://biominas.org.br/blog/technology-readiness-level-trl-2/`. Date accessed: 13-01-2022.

[189] Lars Frølund, Fiona Murray, and Max Riedel. Developing successful strategic partnerships with universities. *MIT Sloan Management Review*, 59(2), 2018. ISSN 15329194.

[190] Charles W. Wessner. *Innovation Policies for the 21st Century: Report of a Symposium*, volume 53. 2007.

[191] Joel R Campbell. Building an IT Economy: South Korean Science and Technology Policy. *Issues in Technology Innovation*, (19), 2012.

[192] Oxford University Press. Definition of suspense in English, 2019. URL https://www.lexico.com/en/definition/suspense. Date accessed: 25-02-2022.

[193] F. Zhao and G.P. Zeng. Innovation ecosystem under multiple perspectives (in Chinese). *Studies in Science of Science*, 32(12):1781–1788, 2014.

[194] S.Q. Chen and L.G. Gu. The analysis of enterprise technology innovation ecosystem (in Chinese). *Science and Technology Management Research*, 28(7):453–454, 2008.

[195] Nicholas W. Fischer, Aaron Prodeus, David Malkin, and Jean Gariépy. p53 oligomerization status modulates cell fate decisions between growth, arrest and apoptosis. *Cell Cycle*, 15(23), 2016. ISSN 15514005. doi: 10.1080/15384101.2016.1241917.

[196] Meerman A. Muros V. Orazbayeva B. Baaken T. Davey, T. The State of University – Business Cooperation in Europe. Technical report, Publication Office of the European Union, Luxembourg, 2018.

[197] Medical Delta. Living Labs, 2019. URL https://www.medicaldelta.nl/living-labs-field-labs. Date accessed: 10-12-2021.

[198] S.R. Davies and M. Horst. Histories: Telling the Story of Where Contemporary Science Communication, This Book, and Our Own Work Come From. In *Science Communication: Culture, Identity and Citizenship*, volume 10.1057/97, chapter 2, pages 29–51. Palgrave Macmillan UK, 1 edition, 2016.

[199] Ruben Vicente-Saez and Clara Martinez-Fuentes. Open Science now: A systematic literature review for an integrated definition. *Journal of Business Research*, 88, 2018. ISSN 01482963. doi: 10.1016/j.jbusres.2017.12.043.

[200] European Commission. *Open innovation, open science, open to the world - a vision for Europe*. Brussels, 2016.

[201] Henry Chesbrough. From Open Science to Open Innovation. Technical report, Institute for Innovation and Knowledge Management, ESADE, Berkeley, 2015. URL https://www.fosteropenscience.eu/sites/default/files/pdf/1798.pdf. Date accessed: 03-03-2022.

[202] European Synthetic Cell Initiative. Hub Zwart: to avoid dual use, steer synthetic cell research towards sustainability, 2021. URL https://www.syntheticcell.eu/2021/10/07/hub-zwart-to-avoid-dual-use/. Date accessed: 06-01-2022.

[203] André Pereira de Carvalho and José Carlos Barbieri. Innovation and sustainability in the supply chain of a cosmetics company: A case study. *Journal of Technology Management and Innovation*, 7(2), 2012. ISSN 07182724. doi: 10.4067/s0718-27242012000200012.

[204] Jens Horbach. Determinants of environmental innovation-New evidence from German panel data sources. *Research Policy*, 37(1), 2008. ISSN 00487333. doi: 10.1016/j.respol.2007.08.006.

[205] data.europa.eu. Flash Eurobarometer 315: Attitudes of European entrepreneurs towards eco-innovation, 1 2015. URL https://data.europa.eu/data/datasets/s904_315?locale=en. Date accessed: 14-01-2022.

[206] Kristel Miller, Rodney McAdam, and Maura McAdam. A systematic literature review of university technology transfer from a quadruple helix perspective: toward a research agenda. In *R and D Management*, volume 48, 2018. doi: 10.1111/radm.12228.

[207] Quantum Delft. Quantum Delft, 2022. URL https://quantumdelft.nl/. Date accessed: 15-01-2022.

[208] Saku J. Mäkinen and Ozgur Dedehayir. Business ecosystems' evolution-an ecosystem clock-speed perspective. *Advances in Strategic Management*, 30, 2013. ISSN 07423322. doi: 10.1108/S0742-3322(2013)0000030007.

[209] Toby Stuart and Olav Sorenson. The geography of opportunity: Spatial heterogeneity in founding rates and the performance of biotechnology firms. *Research Policy*, 32(2 SPEC.), 2003. ISSN 00487333. doi: 10.1016/S0048-7333(02)00098-7.

[210] L.G. Zucker, M.R. Darby, and M.B. Brewer. Intellectual human capital and the birth of US biotechnology enterprises. *American Economic Review*, 88:290–306, 1998.

[211] Uta Kirchner and Agnes von Matuschka. Patent commercialisation. Founder Survey of the Technical University of Berlin 2008/09. Technical report, TU Berlin, Gründungsservice, Berlin, 8 2011.

[212] Annalee Saxenian. International Mobility of Engineers and the Rise of Entrepreneurship in the Periphery AnnaLee Saxenian *. *Development*, 2006.

[213] Jasjit Singh. Collaborative networks as determinants of knowledge diffusion patterns. *Management Science*, 51(5), 2005. ISSN 00251909. doi: 10.1287/mnsc.1040.0349.

[214] Soumaya Ben Letaifa and Yves Rabeau. Too close to collaborate? How geographic proximity could impede entrepreneurship and innovation. *Journal of Business Research*, 66(10), 2013. ISSN 01482963. doi: 10.1016/j.jbusres.2013.02.033.

[215] Bachelor Nanobiology. Detailed study plan, 2022. URL https://d2k0ddhflgrk1i.cloudfront.net/Studentenportal/Faculteitspecifiek/TNW/Onderwijs/Modulekaarten/NB-BSc-kaart.pdf. Date accessed: 17-01-2022.

[216] Master Nanobiology. Summary of curriculum, 2022. URL https://d2k0ddhflgrk1i.cloudfront.net/Studentenportal/Faculteitspecifiek/TNW/Onderwijs/Modulekaarten/NB-MSc-kaart.pdf. Date accessed: 17-01-2022.

[217] TU Delft. Ondernemerschap: MedTech-Based Entrepreneurship, 2022. URL https://www.tudelft.nl/en/tpm/education/minors/ondernemerschap-medtech-based-entrepreneurship. Date accessed: 17-01-2022.

[218] TU Delft. TU Delft Graduate School, 2022. URL https://www.tudelft.nl/en/education/programmes/phd. Date accessed: 16-01-2022.

[219] Einar Rasmussen, Simon Mosey, and Mike Wright. The influence of university departments on the evolution of entrepreneurial competencies in spin-off ventures. *Research Policy*, 43(1), 2014. ISSN 00487333. doi: 10.1016/j.respol.2013.06.007.

[220] UNIIQ. Over UNIIQ, 2022. URL https://uniiq.nl/#over. Date accessed: 16-01-2022.

[221] European Commission. Communication from the Commission to the European Parliament, The Council, The European Economic and Social Committee and the Committee of the Regions: Europe 2020 Flagship Initiative, Innovation Union. Technical report, Brussels, 2010. URL http://ec.europa.eu/research/innovation-union/pdf/innovation-union-communication_en.pdf. Date accessed: 16-01-2022.

[222] European Commission. Horizon 2020: Work Programme 2016-2017. 16. Science with and for Society. Technical report, Brussels, 2016. URL http://ec.europa.eu/research/participants/data/ref/h2020/wp/2016_2017/main/h2020-wp1617-swfs_en.pdf. Date accessed: 16-01-2022.

[223] Sanjay Jain, Gerard George, and Mark Maltarich. Academics or entrepreneurs? Investigating role identity modification of university scientists involved in commercialization activity. *Research Policy*, 38(6), 2009. ISSN 00487333. doi: 10.1016/j.respol.2009.02.007.

[224] European Commission. EIC Transition Open 2021, 2021. URL https://ec.europa.eu/info/funding-tenders/opportunities/portal/screen/opportunities/topic-details/horizon-eic-2021-transitionopen-01;callCode=null;freeTextSearchKeyword=;matchWholeText=true;typeCodes=1;statusCodes=31094501,31094502,31094503;programmePeriod=2021%20-%202027;programCcm2Id=43108390;programDivisionCode=null;focusAreaCode=null;destination=null;mission=null;geographicalZonesCode=null;programmeDivisionProspect=null;startDateLte=null;startDateGte=null;crossCuttingPriorityCode=null;cpvCode=null;performanceOfDelivery=null;sortQuery=sortStatus;orderBy=asc;onlyTenders=false;topicListKey=topicSearchTablePageState. Date accessed: 16-01-2022.

[225] Rijksdienst voor Ondernemend Nederland. Octrooirecht. URL https://www.rvo.nl/onderwerpen/innovatief-ondernemen/octrooien-ofwel-patenten/octrooi-anders-beschermen/octrooirecht. Date accessed: 04-03-2022.

[226] European Patent Office. FAQ - applying for a patent. URL https://www.epo.org/service-support/faq/own-file.html. Date accessed: 04-03-2022.

[227] Anton Nyström. The Loss of Chaos: Figurational Togetherness with Digital Distance Work. Master's thesis, Uppsala, 6 2021.

[228] Eileen Ahlin. *Semi-Structured Interviews With Expert Practitioners: Their Validity and Significant Contribution to Translational Research*. 2019. doi: 10.4135/9781526466037.

[229] Manos Mavrakis, Yannick Azou-Gros, Feng Ching Tsai, José Alvarado, Aurélie Bertin, Francois Iv, Alla Kress, Sophie Brasselet, Gijsje H. Koenderink, and Thomas Lecuit. Septins promote F-actin ring formation by crosslinking actin filaments into curved bundles. *Nature Cell Biology*, 16(4), 2014. ISSN 14764679. doi: 10.1038/ncb2921.

[230] Simon Kretschmer, Kristina A. Ganzinger, Henri G. Franquelim, and Petra Schwille. Synthetic cell division via membrane-transforming molecular assemblies. *BMC Biology*, 17(1), 2019. ISSN 17417007. doi: 10.1186/s12915-019-0665-1.

[231] Debra A. Kendall and Robert C. MacDonald. Characterization of a fluorescence assay to monitor changes in the aqueous volume of lipid vesicles. *Analytical Biochemistry*, 134(1), 1983. ISSN 10960309. doi: 10.1016/0003-2697(83)90258-0.

[232] Geert Van Den Bogaart, Matthew G. Holt, Gertrude Bunt, Dietmar Riedel, Fred S. Wouters, and Reinhard Jahn. One SNARE complex is sufficient for membrane fusion. *Nature Structural and Molecular Biology*, 17(3), 2010. ISSN 15459993. doi: 10.1038/nsmb.1748.

[233] Marten Exterkate, Antonella Caforio, Marc C.A. Stuart, and Arnold J.M. Driessen. Growing Membranes in Vitro by Continuous Phospholipid Biosynthesis from Free Fatty Acids. *ACS Synthetic Biology*, 7(1), 2018. ISSN 21615063. doi: 10.1021/acssynbio.7b00265.

[234] Patricia C. Weber, D. H. Ohlendorf, J. J. Wendoloski, and F. R. Salemme. Structural origins of high-affinity biotin binding to streptavidin. *Science*, 243(4887), 1989. ISSN 00368075. doi: 10.1126/science.2911722.

[235] Thermo Fisher Scientific. His-tagged Proteins – Production and Purification. URL https://www.thermofisher.com/nl/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/his-tagged-proteins-production-purification.html. Date accessed: 22-02-2022.

[236] Loubna El Harrad and Aziz Amine. Chronoamperometric Biosensor for Protease Activity Assay and Inhibitor Screening. *Electroanalysis*, 29(10), 2017. ISSN 15214109. doi: 10.1002/elan.201700340.

[237] Marjorie L. Longo and Hung V. Ly. Micropipet aspiration for measuring elastic properties of lipid bilayers. *Methods in Molecular Biology*, 400, 2007. ISSN 10643745. doi: 10.1385/1-59745-519-9:421.