Influence of Assay Parameters on Structural and Functional Studies of Membrane Proteins

Flip Bastiaan Jansen

Bachelor End Project

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Supervisor: Dr. Duncan G.G. McMillan
Daily Supervisor: Albert Godoy-Hernandez
Duncan McMillan Group
Department of Biotechnology
Delft University of Technology, Delft

Preface

Due measurements taken to fight the spread of SARS-CoV-2 I was unable to continue with my initial BEP project of quantifying quinone and lipid composition in *E. coli* extracted using varying detergent concentrations. This, since lab access was not an option during my thesis period. As an alternative BEP implementation I participated in the Corona Super Research Project (CSRP) and wrote a review on a subject similar to my original BEP proposal. Both the review and entry into the CSRP are incorporated into this BEP document.

Aknowledgement

I would like to thank my supervisor Duncan McMillan and daily supervisor Albert Godoy Hernández for guiding me throughout period of my thesis and their continued effort of supporting my research and driving inspiration in spite of the corona virus pandemic.

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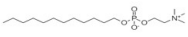
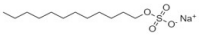
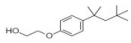
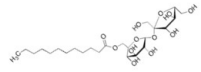
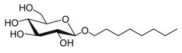
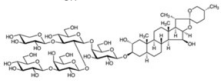
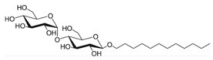
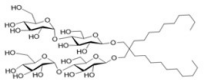
Detergent Table

Chemistry	Detergent	Structure	Type	Tm (°C)	CMC (mM)	Aggregation number	Micelle size (kDa)	Shape
Phosphocholine	Fos-Choline 12		zwitterionic	224-226[D]	1.5[A]	~54[C]	19[A]	conical
sodium laurilsulfate	Sodium dodecyl sulfate (SDS)		anionic	204-207[D]	3 [21]	~80[21]	23[21]	conical
Polyoxyethylene	Triton-X		nonionic	65[E]	0.23[A]	75–165[C]	60–90[B]	cylindrical
Sucrose esters	Sucrose monolaurate		nonionic	150-152[22]	0.3[E]	35-99[22]		cylindrical
Glucopyranoside	n-Octyl-β-D-glucopyranoside (OG)		nonionic	105[D]	18[A]	30–100[C]	19[A]	cylindrical
Saponin	Digitonin		nonionic	230-240[D]	0.25–0.5[B]	~60[E]	70-75[B]	cylindrical
Maltoside	n-Dodecyl-β-D-maltopyranoside (DDM)		nonionic	224-226[D]	0.17[A]	80-150[C]	59.5[A]	inverse-conical
Maltose NG	Lauryl maltose neopentyl glycol (LMNG)		nonionic	57[23]	0.01[A]	~400[B]	91–393[B]	inverse-conical

In Brief

Detergents strongly influence the properties of membrane proteins, potentially having a structural role on detergent-solubilized enzymes. We often need detergents to isolate membrane proteins, for biochemical and structural characterization. A review on the influence of chancing different parameters in detergent facilitated membrane protein extraction/solubilization/purification/reconstitution.

Highlights

- Membrane Protein Physiology
- Protein-Detergent Complexes (PDCs)
- Assay Parameters

Influence of Assay Parameters on Structural and Functional Studies of Membrane Proteins

F.B. Jansen

Abstract

Structural and functional characterization of membrane proteins (MPs) requires extraction from the native lipid bilayer, which generally necessitates the formation of water-soluble MPs structures or complexes suitable for biochemical assays. These structures are often detergent micelles that form protein-detergent complexes (PDCs), due to detergents being straightforward in implementation and providing homogenous yields. However, obtaining physiologically relevant PDCs remains a challenge, because most detergents do not keep the native structure/function of the protein intact, jeopardizing its stability, and disrupting the proton pressure and electric gradient even after reincorporation in artificial lipid membranes. To achieve native-like physiology (i.e. to maintain native protein-associated lipids and structurally relevant ligands or cofactors), it is essential to choose the right extraction parameters. Nevertheless, the field of membrane biochemistry is still missing a holistic, interdependent understanding of these systems, too often neglecting the evident, key role that detergents play. In this review, we aim to obtain a more fundamental understanding of detergent-solubilized membrane proteins by pinpointing the most relevant assay parameters (i.e. the most influential on the biochemical properties) involving MPs.

Keywords: membrane proteins; detergents; functional-structural assays; physiology

1 Introduction

MPs make up a significant part of the functional human genome; roughly a quarter of the human proteome is MP related [1],[2]; thus, their importance in cell and organism function is not to be understated. MPs perform a multitude of functions: from signal transduction [3] to particle transport [4], - and energy conversion, e.g. using ATP synthases [5]. This importance is reflected in that MPs are estimated to make up well over half of all pharmaceutical drug targets [6]. Surprisingly, MPs only make up a fraction of mapped protein structures, at approximately 2%. However, this is explained by the difficulties associated with obtaining a homogeneous and soluble protein sample, needed for characterization and, consequently, inclusion in such genomic maps. Acquiring a homogenous and soluble sample while maintaining in vivo properties is a persistent challenge for the study of MPs, as a result of the intrinsic complexity of said in vivo environment.

The advent of cellular life was inextricably dependent on the formation of a functional barrier between the intracellular and extracellular environment, the biological membrane. While its initial purpose may have been for segregation, and indeed the more complex the life form, the more membrane compartments the cell has, they have since been repeatedly proven to a wide-ranging role in biological processes. As a barrier they allow the concentration of metabolic solutes, proteins, and ions, however in doing so they keep proteins functionally close enough to conduct essential biological processes. However, in performing this function they also facilitate the exchange and active transport of materials, conduct complex interactions, interpret extracellular conditions, and are the first part of the cell to adapt to environmental changes. To conduct all of these processes the membrane must be dynamic in both physical and biological nature. Therefore, a cellular membrane is made up of a multitude of different components, the basic element being a lipid bilayer. MPs are associated with the hydrophilic external interface of the lipid bilayer (peripheral membrane proteins: PMPs), or span the lipid bilayer and transverse the hydrophobic interior (integral membrane proteins: IMPs), as is illustrated in Figure 1. Both PMPs and IMPs are part of a very heterogeneous environment built from a remarkable variety of lipids. Considering the diversity of protein shape and movement, this is a fact that has been largely ignored, yet may drastically dictate the physiological properties of MPs.

A significant underlying reason for not reaching a more fundamental understanding of the process of assaying these MP's is that many factors are involved. In trying to achieve a better understanding, certain critical factors are often overlooked, such as the detergent concentration at different stages

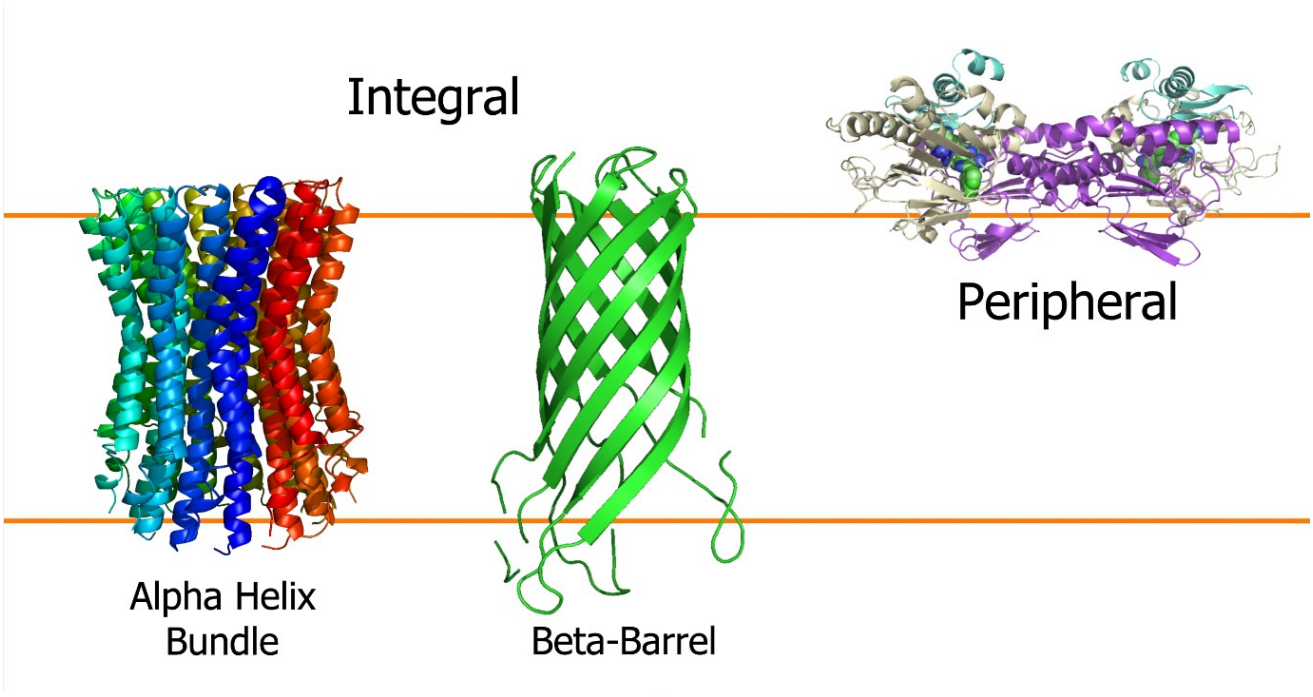


FIGURE 1: Indication of structural shapes of both peripheral and integral MPs relative to the cell membrane - which is illustrated as the region between the two orange lines. Individual proteins were rendered using Pymol (Delano Scientific).

of the assay, the influence of extensive washing with detergent, changes in the melting temperatures (T_m) of proteins and PDCs, and the influence of bio beads used in columns among others. For this reason, this review examines the importance of the above-mentioned parameters, or more generally, the influence of detergent type/concentration/exposure time, the temperature of the assay, and specific considerations for obtaining physiological relevance. However, before addressing possible influences of assay parameters we first provide the context of the cell environment and its physiological considerations.

1.1 Non-optimal activity

A more general consideration for physiological relevance and interpretation of assays results can be found in the leakiness or perceived non-optimal activity of proteins and complexes, which is most often a function of the plasticity of the system or the extent to which a system can adapt to varying conditions. Or otherwise stated, in nature, the chaotic changing of environments values the property of anti-fragility, which coincides with the plasticity of functioning, and in turn prefers the non-optimal functioning of systems to allow a wider range of operating conditions. A good example being the measured long-lifetime proton leak state in cbo_3, a heme-copper oxidase [7]. In line with the reasoning for increased operational plasticity, is the perceived sub-optimal operation of the protein in the form of proton leakage in small sub-populations of heme-copper oxidases in a living organism, which might help to alleviate respiratory pressure and factors related to reactive oxygen species production. Conversely, in solution-based assays, this proton leak state is not observed. The physiological relevance of the solution-based assay result is less important when the end goal of biocatalysis is concerned. However, if the assay goal is to study biology, then physiological relevance is very much of importance, leading to perceived sub-optimal function to be more of an expected outcome, which is something to consider when evaluating the results of an assay.

2 Properties of the lipid environment and interactions with its Denizens

Having a multi-component, highly heterogeneous lipid bilayer environment, combined with the fact that most MPs are present in low-density among many others, means that an efficient extraction method is needed for a homogeneous, comprehensive sample. The native lipid bilayer provides structural rigidity, protection of hydrophobic parts against exposure to aqueous solutions, and maintenance of an ionic pressure gradient, all of which contribute to MP stability in the in vivo environment. Problems arise when the resulting structure after MP extraction does not offer the same physiological characteristics of the native lipid bilayer, this is, unfortunately, the case for most extraction methods

and therefore necessitates the conservation of native MP components and state. This includes the preservation of native lipids, ligands, and co-factors.

2.1 Importance lipids for MP structure and function

Loss of natively associated lipids trough exposure to detergents is apparent to be instructive in causing perturbations of MP structure and function, which is the result of these lipids being structurally and functionally relevant [8]. This importance is reflected through a multitude of different influences. Dowhan Bogdanov observe that lipids influence MP topology, which is of key importance for proper protein function [9]. Moreover, Palsdottir and Hunte argue that: "Tightness and specificity of lipid binding to bacteriorhodopsin is indicated by the fact that only native lipids are retained during purification and observed in the structures regardless of different experimental approaches and crystallization conditions." They observe that these lipids bound to specific residues of the protein maintain a sort of seal on the protein, which functions to prevent compromise of the electrical gradient the native bilayer maintains. Palsdottir and Hunte found that conservation of lipid-enriched binding sites was present across species boundaries, indicating some functional importance upholding cross-species conservation. An example supporting the claim of functional importance of lipids is the finding of Costeira-Paulo *et al* that lipids - phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), and cardiolipin (CDL) - direct structure and function of the electron acceptor-binding site of a peripheral membrane protein dihyroorotate dehydrogenase [10]. In essence, these natively associated lipids function in a matter analog to an exoskeleton - they provide structure, protection, and sealing of the protein. These lipids are stripped when the protein is extensively exposed to detergents, which is often the case when assay consists of repeated washes and or columns using detergents. Supportive of this indicated importance of lipids is the influence the lipid environment has on the protein-protein interaction in the bilayer [11]. Research with the difference in native lipids present as the focal point reaffirms the importance of these lipids. An illustrative example is the light-harvesting complex II, where it has been shown that the assembly depends on these natively associated phospholipids and glycolipid species [12].

2.2 Lipid headgroup-to-tail size ratio differences

Lipid shape is one the defining features of a biological membrane and must be considered extremely carefully before thinking about the study of any membrane protein in any artificial membrane platform; yet is a comparatively rarely addressed matter among the studies of membrane proteins in the field of biochemistry. The shape of a membrane lipid depends on the area its polar head occupies, relative to the dimensions of its hydrophobic fatty acid tail moieties [13], [14]. Membrane lipids display either a cylindrical shape (e.g. phosphatidylcholine and phosphatidylserine), a conical shape (e.g. phosphatidylethanolamine and phosphatidic acid), or an inverted conical shape (e.g., glycolipids, lysolipids). The two most distinct groups are the polar lipids and glycolipids, which have a conical and inverse conical headgroup-to-tail size ratio respectively. Both these groups are indicated to have structural importance, but using distinct modes of actions as a consequence of this differing molecule shape, an example of lipid-type dependent function can be observed in the increased presence of glycolipids, like DGDG, in thylakoid membranes of photosystem II [15]. These headgroup-to-tail size ratios dictate possible protein surface positions the molecule can occupy, where a high ratio and low ratio present distinct shapes, favoring occupying distinct positions. This principle applies to amphiphilic molecules and therefore also to detergents, which provides an additional relevant detergent property, this shape determination is illustrated in Figure 2. Moreover, this distinct amphiphile placement preference is driven by the entropic effect of the particle's respective hydrophobicity. This hydrophobicity is driven by either increasing the effective tail size, which increases the hydrophobicity of the molecule, or conversely, increasing effective headgroup size, which decreases the hydrophobicity.

Similarly to lipids, proteins also influence the shape of a membrane creating areas of intensified thickness (i.e. in lipid rafts) and these polymorphisms influence the localization of lipid molecules within the biological membranes. Even though the shape of integral membrane proteins is a major player in the curvature of membranes, lipid shape also plays a critical role. Therefore, the asymmetry of lipid distribution in each leaflet is critically important. Moreover, Lipid polymorphism not only induces lipid asymmetry between the two leaflets of membranes, but it is also responsible for phase separation within one monolayer leaflet. For example, it is assumed that lipid polymorphism is involved in the formation of lipid rafts, which are enriched in sphingolipids and cholesterol [16]. Due to its conical shape, cholesterol may play the role of molecular spacer to fulfill the free space between sphingolipid molecules, which exhibit an inverted conical shape.

Another parameter of importance is the assay temperature, a consideration for which can be found in the melting temperature (T_m) of the natively associated lipids/ligand(s), MP, detergent, and notably the combined system T_m – where it has been shown that individual components influence each other's respective T_m. An example of such a study being the observed varying T_m of detergents

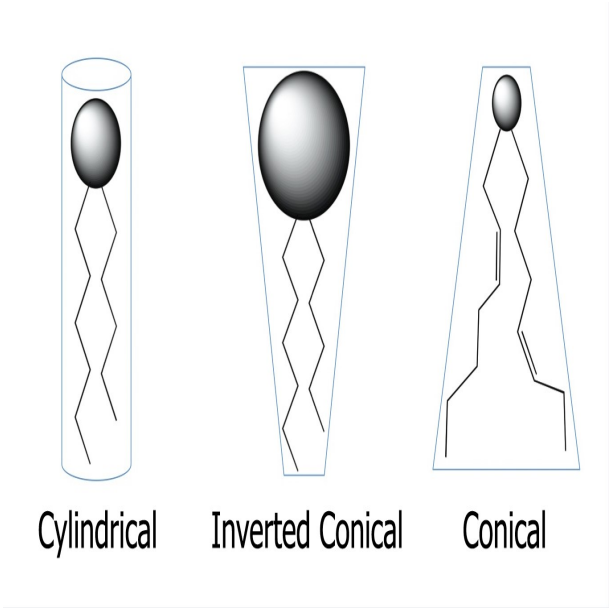


FIGURE 2: The various lipid phase shape found in biological membranes, where the difference in headgroup-to-tail size ratio results in differing effective amphiphilic structures. In order of appearance from left to right: an even head group/tail size and cylindrical shape, a high headgroup-to-tail size ratio and inverse conical shape, and a low headgroup-to-tail size ratio leading to a conical shape.

based on the presence of different MPs [17]. Importantly, the combined T_m of the local environment is the feature of importance [18] since this dictates the viscosity the MP experiences, as a specific viscosity facilitates a certain freedom of movement - necessary for native structure and function [19]. Moreover, the right assay temperature and resulting viscosity are also required for lipids to be correctly associated with the MP [20]. This way, non-physiological temperatures that do not take into account the native T_m of the local environment or PDC can result in loss of and failure to restore natively associated lipids, which in turn causes undesired perturbation of the MP structure and function. Currently, most assays take into account the T_m of the protein of interest. However, the T_m of other critical components like the natively associated lipids or used detergent, let alone the T_m of the combined system, are often not taken into account. This can create a scenario where an assay temperature is chosen which might be optimal for the MP of interest but outside the effective domain for the associated lipid/ligand(s) and used detergent. This can result in unaccounted for mechanisms influencing assay outcomes, such as denaturation or rate-limiting viscosity of previously mentioned components. An example of the influence of essay temperature relative to T_m being the differences in measured C. thermarum NDH-2 activity at 65 °C (native T_m) while using E. coli polar lipids liposomes (native temperature of 37 °C) [21]. Therefore, future considerations of an MP assay could benefit from taking into account the T_m of all relevant substance components-combinations and the local native environment of the sample for said assay.

Cannon *et al* observe that changing of the lipid composition leads to varying MP activity, as is indicated in Figure 3 [22]. This seems to indicate that specific lipids bind to/associate with different locations on the MP, a-kin to a certain MP lipid specificity, which is further supported by the finding that the lipids most commonly used in MP assays, POPC, do not fill the same nooks and crannies that the native lipids do. The assumption is that different specific lipids induce MP specific variations in measured activity. Asseri *et al* confirm this by observing that adding cardiolipin affected activity in cytochrome bd and bo_3 from E.coli [23]. Conversely, Nilsson *et al* found that using liposomes primarily made up of POPC with incorporated cytochrome bo_3 and ATP synthase actually resulted in relatively higher ATP synthesis activity by proton pumping as compared to adding more native lipids, like cardiolipin, also indicating activity regulation trough lipid type [24]. However, in the case of POPC regulated activity, one can ask the question of whether this biocatalysis result is relevant for native physiology since POPC is not a naturally occurring lipid.

3 *In vogue* detergents and alternatives for membrane protein handling

To independently assess MP structure and function requires the protein to be removed from the native bilayer environment - through extraction and subsequent purification. Historically, detergents have been the most used tool for MP extraction and subsequent analysis [25]. Moreover, detergents still are the most effective and reliable method for structural or functional analysis. Detergents are amphiphilic molecules that facilitate the blending of hydrophobic mixtures in water, i.e. extracting and solubilizing MPs. They consist of a hydrophilic head group and a hydrophobic ‘tail group’ in a similar arrangement to the general lipid structure. In contrast to the relatively few known MP structures, there is a vast choice of available detergents (a selection of relevant detergents and associated properties are indicated in Table 1). Even though using detergents for extraction, solubilization, or reconstitution of these MPs is a simple to implement a process, it does have its limitations. Considerations when choosing the right detergents are dependent on the class and group of the chosen detergent.

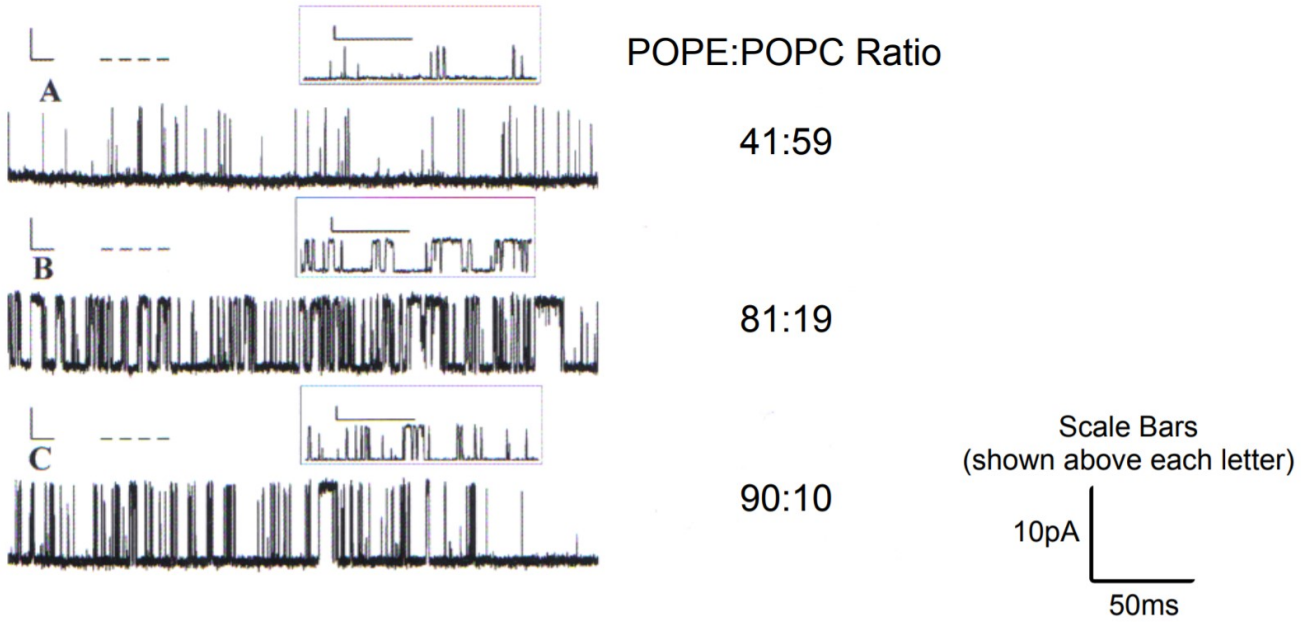


FIGURE 3: Showing gating activity versus time plots of single RyR/Ca2+ channel reconstituted into varying POPE/POPC ratio bilayers (41:59 for A, 81:19 for B and 90:10 for C). The axes for current and time are 10 pA and 50 ms, respectively [22].

3.1 General Detergent Properties

In aqua, detergents form micelles: single-layer porous structures. When a membrane protein is in solution these micelles are present alongside protein-detergent complexes (PDC's), when the concentration of the detergent is above a specific concentration. This concentration is very specific to the chemistry of the detergent and is known as the 'critical micelle concentration' (CMC) which depends on the monomer aggregation number (N) and dictates micelle size. Longer or bulkier alkyl-chained detergents often have lower CMCs, indicating a larger energetic favorability to form micelles. Moreover, increased effective alkyl chain size is most often indicative of a higher N and larger micelle size. The aggregation number increases by 16 per alkyl carbon added (on average). Conversely, increasing the steric bulk of the lipid head-group has the opposite effect [26]. These larger/bulkier detergent monomers form larger micelles with higher N that aggregate at lower concentrations, indicative of a lower CMC. In assays that require altering detergent concentration or detergent types, a higher CMC is preferred, since, detergent micelles with a high CMC tend to disassemble when the detergent concentration is lowered trough either dialysis or dilution. For low-CMC detergents, removal using bio beads or size exclusion offers an alternative. However, these methods have their complications, as will be discussed subsequently. The size of the micelles and their tendency to aggregate is indicative of the structural rigidity and stability of the micelle. Larger size and a low CMC is most often indicative of more stable and rigid micelles [27]. These are important considerations when protection of structure and function of the incorporated MP is concerned.

Detergent head groups may be charged or neutral and vary wildly in physical size. Charge status influences MP extraction and purification, via interactions between the detergent molecules and the neighboring charged residues of the MP. However, this often contributes to increased protein denaturation by breaking quaternary protein-protein bonds [28], explaining why non-ionic detergents, which have no charge, are better suited and preferred for MP analysis. For most biological assays there are 3 main detergent groups, these are the anionic, non-ionic, and zwitterionic detergents. The group names are indicative of the charged state of the detergent monomer, where anionic means the monomer contains an alkylbenzene headgroup which is lipophilic/hydrophobic and a hydrophilic sulfonate residue. A historically often used detergent of this class is the harsh but high yield sodium dodecyl sulfate (SDS). Zwitterionic detergents have headgroups with an equal number of positively and negatively charged functional groups, with a net zero charge. Fos-Choline-12 (FC12) is an often-used example of a zwitterionic detergent, which is a detergent similar to SDS. Non-ionic indicates that the headgroup is completely uncharged, detergents such as Triton-X and detergents with a maltoside headgroup, like DDM and LMNG, belong to this latter group.

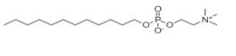
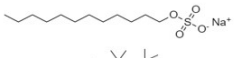
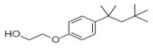
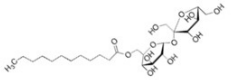
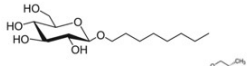
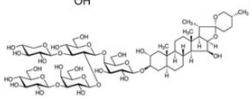
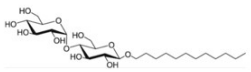
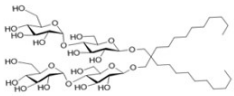
Chemistry	Detergent	Structure	Type	Tm (°C)	CMC (mM)	Aggregation number	Micelle size (kDa)	Shape
Phosphocholine	Fos-Choline-12		zwitterionic	224-226[D]	1.5[A]	~54[C]	19[A]	conical
Sodium laurilsulfate	Sodium dodecyl sulfate (SDS)		anionic	204-207[D]	3[21]	~80[21]	23[21]	conical
Polyoxyethylene	Triton-X		nonionic	65[E]	0.23[A]	75–165[C]	60–90[B]	cylindrical
Sucrose esters	Sucrose monolaurate		nonionic	150-152[22]	0.3[E]	35-99[22]		cylindrical
Glucopyranoside	n-Octyl-β-D-glucopyranoside (OG)		nonionic	105[D]	18[A]	30–100[C]	19[A]	cylindrical
Saponin	Digitonin		nonionic	230-240[D]	0.25–0.5[B]	~60[E]	70-75[B]	cylindrical
Maltoside	n-Dodecyl-β-D-maltopyranoside (DDM)		nonionic	224-226[D]	0.17[A]	80-150[C]	59.5[A]	inverse-conical
Maltose NG	Lauryl maltose neopentyl glycol (LMNG)		nonionic	57[23]	0.01[A]	~400[B]	91–393[B]	inverse-conical

TABLE 1: Commonly used detergents in biological assays, indicating: group name, general name, structure, type, melting temperature (T_m), critical micelle concentration (CMC), micelle detergent monomer aggregation number, micelle size in kDa, and detergent shape - dictated by the headgroup-to-tail size ratio. Detergent structures were made using the ChemDrawdirect web application. Source indicated as superscript A for [17], B for [29], C for Anatrace, D for the Chemicalbook, E for Sigma Aldrich, and other individual sources: [30],[31],[32]

3.2 Selected Contemporary Detergent Properties

Given the plethora of detergents both on the market and under development, we chose to only focus on those which have been used the most in various studies on membrane protein structure-function, this is certainly not an attempt at exhaustive coverage but covers the core detergents of contemporary interest.

Triton X-100 (TX100) or octyl phenol ethoxylate is generally viewed as a harsh detergent but is conversely considered extremely useful for archaeal membranes and extremophile membranes, which are often highly adapted and 'non-conventional' in lipid structure. TX100 has a CMC of 0.23 mM [17] and larger average micelle size of 60-90 kDa [29].

In contrast, Digitonin, a saponin, is an extremely gentle non-ionic detergent well suited for isolating supercomplexes. However, to date, there have been no reports of successful reconstitution after solubilization using Digitonin. Digitonin has a relatively low CMC of 0.5 mM and therefore a larger average micelle size of 75 kDa [29]. Glyco-diosgenin (GDN) is a synthetic variant proposed to circumvent the toxicity of the naturally occurring digitonin. Moreover, DGN provides less batch to batch variation than naturally occurring digitonin. n-Octyl--D-glucopyranoside (OG) is also a non-ionic detergent slightly less mild than Digitonin due to it being more hydrophobic as a whole. OG is one of the most used glucoside-based detergents. Its headgroup has one less sugar moiety maltosides. It has a high CMC of 18 mM and therefore has a rather compact micelle with a size of 19 kDa [29].

n-Dodecyl--D-maltopyranoside (DDM) is currently the most used detergent for MP Assays [29]. DDM has a hydrophilic maltose headgroup and a hydrophobic alkyl tail, thus part of the class of alkyl maltosides. DDM is non-ionic with a slightly hydrophobic tail and is therefore considered a mild detergent able to effectively extract many proteins in a stable state. Since DDM has a low CMC (0.0087%/0.17mM) [29] less detergent is required for retaining the extracted membrane protein in a monodispersed state. On the other hand, this low CMC means that extracting said detergent for reconstitution through dialysis or dilution is not feasible. Moreover, DDM has a very low CMC which causes its detergent micelle to be rather large (Mw 70 kDa) [29].

Lauryl maltose neopentyl glycol (LMNG) is seemingly the new challenger to DDM and it currently is the detergent of choice for membrane protein crystallization [29]. It is synthesized by crossing at the Lauryl carbon of two DDM molecules, as will be further discussed subsequently. LMNG has a very low CMC of 0.01 mM which is reflected by a

relatively large micelle size of around 200 kDa [29]. The crossed structure is more rigid than DDM and combined with the other features this provides a more stable structure where the MP has less chance of being inactivated. Additionally, the similarity of the double- polar headgroup and globally apolar tail structure of LMNG as compared to specific lipids, such as cardiolipin, enables LMNG to occupy places and support the MP, in a way that other detergents might not be able to. Therefore, LMNG can associate with the MP in matter more closely resembling native physiology. Each detergent type has its specific conditions favoring said detergent. However, in most use cases LMNG is indicated to have the best-suited characteristics. Especially, in its relative ability to maintain native structure, by increased preservation of native lipids and associated enzymes [33]. This can be seen as a consequence of LMNG's distinct headgroup-to-tail size ratio, which differentiates LMNG from other detergents, with its relatively large headgroup and smaller effective tail size. Consequently, this headgroup-to-tail size ratio dictates possible protein surface positions the detergent can occupy, where a high ratio and low ratio present distinct shapes, favoring occupying distinct positions. it follows that LMNG, with its opposite ratio structure to lipids, such as cardiolipin (LMNG inverse conical and cardiolipin conical), does not compete to occupy the same positions into the protein surface. Thereby, leading to the preservation of these lipids in LMNG facilitated extraction. Moreover, exchanging LMNG for DDM in DDM-extracted proteins does not fully recover protein activity to levels of LMNG-extracted proteins [34]. Indicating a possible permanently denaturing effect of DDM that further establishes the beneficial nature of LMNG facilitated extraction, over DDM, and most likely other detergents as well.

3.3 Alternatives to conventional Detergents

Current methods that try to improve on conventional detergents are being researched and applied with an effort to improve on existing detergent methods by trying to minimize the previously mentioned known problems associated with their use. Specifically, methods that employ alternative detergents or assay structure, where the amphiphilic agents have been designed with the intent of solving the issues of conventional detergents, some prime examples being:

Lipopeptide detergents (LPDs) are a class of amphiphile consisting of two alkyl chains supported by a peptide scaffold, where on both sides the chains end on an -helix. LPDs form small micelles of 30 kDa [35] and are very mild non-denaturing which tend to preserve the structure of the MP [36]. Colic acid-based facial amphiphiles (FAs) can also be used as part of a more non-denaturing detergent [37]. However, for the desired micelle characteristics cholic acid-based FAs need to be modified for increased amphiphilicity [38]. Therefore, besides being difficult to use in many parts of the assay, they are also labor and resource-intensive. In contrast, Calixarene-based ionic surfactants are macrocyclic oligomers made of phenol components link trough methylene bridges. Calixarene can be used to function as a surfactant with several attractive micelle properties in the context of MP assays. Specifically, a low CMC at around 0.5 mM and monomer exchange rate [39], contributing to a more stable micelle environment of incorporation of an MP.

Aside from trying to attenuate the negative effects of detergent facilitated extraction/solubilization by modifying the detergent solution, other solutions circumvent the use of detergent altogether. Examples of these techniques that allow detergent free analysis include Styrene maleic acid (SMA) copolymers, which are amphipathic molecules that can insert themselves into the lipid bilayer and can solubilize MPs into a nanodisc shape [40]. The benefit of these SMA nanodiscs is that they provide a near-native environment. Limitation in the use of nanodiscs lays in the incompatibility with several downstream MP assays, such as the reconstitution of the MP with its natively associated lipids [41]. Figure 6 is a schematic representation of the synthesis of SMA.

Other more chemical approaches include the use of (Hemi) fluorinated surfactant ((H)FS), which incorporates fluorine atoms into its hydrophobic tail resulting in lipophobic behavior. Therefore an (H)FS does not function as a detergent. However, under certain conditions, this is used to its advantage. Since certain conditions allow (H)FS to solubilize MPs without having the protein perturbing nature of detergents. Therefore making for an alternative method for solubilization. The specific conditions required for fluorinated surfactant facilitated solubilization currently limit the extent and usefulness of application [42]. Lastly, ionic strength-based solubilization and purification allow isolation of an MP without the use of detergents, an example of an application of this method being the assaying of NDH-2 from the human pathogen *Staphylococcus aureus* [43], [44].

The problem with all these mentioned methods is that they currently either are very labor and resource-intensive compared to the use of a conventional detergent such as LMNG or are only applicable for specific MPs and specific assays. Moreover, most of these detergent-free methods do not allow solubilization of an intact MP like a detergent does, therefore not fully substituting detergent facilitated assaying of MPs. Also, another problem lays in the current difficulty associated with the reconstitution of MPs using these methods, with the exemption in the use of SAM. Therefore, making it unlikely that these methods will fully replace conventional detergents any time soon.

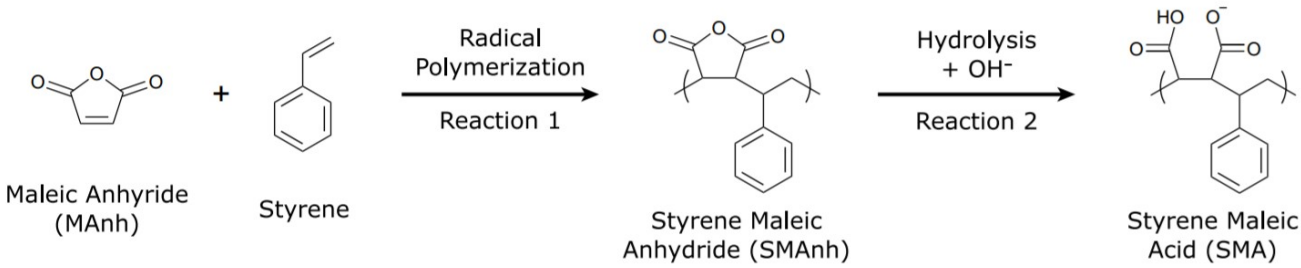


FIGURE 4: Schematic representation of the synthesis of SMA in 2 reaction steps. Firstly, the synthesis of styrene-maleic anhydride (reaction 1), and secondly, the preparation of styrene-maleic acid (reaction 2) [41]

4 The careful considerations of membrane protein purification

Detergent facilitated MP extraction is only effective when detergents are chosen within a limited range of their respective hydrophobicity, which on its own already requires carefully balancing with the rest of its properties. MPs with large hydrophobic outward-facing residues have a stronger tendency to aggregate in solution and therefore require more hydrophobic detergents to match these groups and prevent eventual precipitation of the protein [17]. Moreover, the bulkiness of the detergent, concerning the size/influence of its hydrophobic moieties, with its corresponding thickness of the bound detergent layer, directly correlates with protein stability [45]. On the other hand, these 'stronger' detergents also energetically favor detergent-protein interactions over certain protein-protein interactions causing loss of quaternary structure, resulting in protein denaturation. Therefore, choosing the right detergent balances the need for increased detergent hydrophobicity to prevent aggregation, and not being overly hydrophobic as that could cause denaturation of the MP. Initial detergent screening, therefore, benefits from the knowledge on the proneness of the protein to aggregate or denature.

A potential introduction of uncertainties lies in the lack of detergent concentration measuring capabilities for different stages of the assay. Currently, mostly the initial detergent concentrations are addressed but the influence of specific assay processes on detergent concentration is rarely regarded, which is problematic since it has been established that changes in detergent concentration affect assay outcome [46]. An example where unknown variability in detergent concentration might be introduced is as a consequence of the changes in the amount of detergent that might be lost or stay bound after said size exclusion chromatography, the extend of which is dependent on the detergent and protein present and will therefore vary from assay to assay [47]. This, combined with the repeated dilution of the sample and this means that for the same assay, the amount of detergent present will vary from experiment to experiment; causing experiments to become irreproducible and understanding of the mechanisms involved obfuscated since one cannot interpret the results correctly without knowing all the relevant parameters.

When one wants to remove excess detergent from an assay, whether that be for reconstitution or other purposes, bio beads (polystyrene beads) are an often preferred solution. However, the extent to which these beads interact with lipids and the residual components is poorly understood, and rarely taken into account. Even the initial studies using bio beads do not mention or regard the possibility of bio beads interfering with lipids and altering lipid composition. These articles explored the applications of bio beads and their desired ability to remove detergent while leaving the sample unaffected. This was achieved by probing the effects of these bio beads, i.e. on their ability to remove the detergent and observing any undesired interactions with the protein present [48], [49], [50]. It was concluded that undesired bio bead interactions where negligible, however, this was achieved by observing the bio beads absorption of /interactions with large proteins, like bovine serum albumin [48], based on the difference between pre-column and final concentrations. What is problematic is that these large proteins are not representative of the possible interactions the beads could have with much smaller molecules, such as lipids. One could argue that finding large proteins to be unaffected by these beads compared to their desired interaction with small detergent monomers is not explanative for the bio beads assumed specificity. Since none of the original bio beads application papers address the possible unaccounted for bio beads interference with the sample, it necessitates future research, specifically the interaction with lipids - which as mentioned previously are a key component for maintaining physiological relevancy. Especially since there have not been any studies quantifying the induced alterations of

lipid concentration and composition after application of bio beads. Another possible issue with the application of these bio beads is that columns are known to induce sheer forces, which could alter MP structure. Here, the current problem at hand is the assays resolution of observation, which is limited and not allowing for quantification of such possible dynamics influencing the assay.

In trying to obtain more suitable detergents for extracting and solubilizing MPs, Chea *et al*. required detergent which provided a more stable micelle structure, therefore seeking a detergent with attenuated exchange kinetics and/or strengthened interactions with membrane protein surfaces [51]. This was achieved by increasing the surface as well as the rigidity of their hydrophobic tails. These considerations were taken into account when Chae *et al*. designed the class of detergents of maltose neopentyl glycols (MNG), among which MNG3 or Lauryl Maltose Neopentyl Glucoside (LMNG) has been the most effective member [52]. The way Chae *et al* achieved this increased rigidity of the hydrophobic moieties was by designing the detergent with a crosslink at the lauryl carbon, where the central carbon binds two individual DDM molecules - in between the headgroup and the hydrophobic tail - to form LMNG. LMNG improves on the already mild DDM in that it is even better at maintaining native protein structure and activity while being an effective detergent. This is reflected by an increased activity after extraction and purification using LMNG compared to DDM [52]. Moreover, the choice of detergents seems to not only influence MPs during purification but also in the process of extraction and solubilization, where detergents exert influence on MP physiology [53]. This lasting impact of detergents on MPs is also present in that it is not possible to fully recover the activity from DDM-solubilized cbo_3, even if DDM is exchanged for LMNG [34]. This is most likely the result of the loss of natively associated lipids and/or breaking of quaternary bonds, during exposure to DDM. Even though LMNG presents a more stable and protective PDC, it is not capable of restoring those lost lipids or integral subunit protein-protein bonds. This means that choosing the most well-suited detergent, starting from extraction, is of key importance. The general order of processes required for either a functional or structural MP study, with detergent removal steps for these respective processes, is represented as a flowchart in Figure 5.

Even though LMNG has the beneficial nature of attenuated competition with native lipids due to its difference in structure, it conversely does have similarities in its structure that other detergents lack. Compared to these phospholipids - the lipids that comprise the bilayer - LMNG provides an additional argument for the beneficial detergent characteristics, relative to other detergents. This since, phospholipids, like detergents, are amphiphilic, but instead made up of two hydrophobic tails and a hydrophilic headgroup. This difference in tail number causes these amphiphilic monomers to energetically favor distinct structures: single layer micelles for detergents and a bilayer for phospholipids [54]. The influence of detergent structure on energetic favorability of composite state revalidates the design of the structure of LMNG in a different mater than previously stated. Not only does the LMNG structure provide more rigid hydrophobic moieties and competes less with natively MP associated lipids, but also it more closely resembles the structure of the lipids comprising most of the MP native bilayer environment; something that can be considered to contribute to a more physiologically relevant assay.

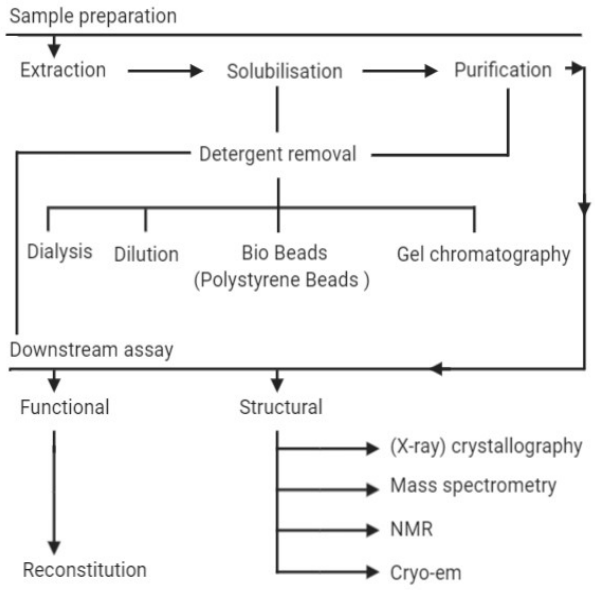


FIGURE 5: Flowchart indicative of the general path of involved processes in an MP assay scheme, with special emphasis on the methods for detergent removal. With these detergent removal methods being employed in nearly all processes involved in an MP assay.

5 Illustrating purification considerations using a cytochrome *bo_3* case study

To illustrate the possibly overlooked aspects of detergent facilitated MP assaying, we studied the clearly defined case study of the cytochrome bo_3 (cbo_3) from *Escherichia coli*, an extensively studied MP. Cbo_3 is a member of the heme-copper superfamily of proton-pumping respiratory oxidases that naturally harbors one-two quinone co-factors. Cbo_3 is very demonstrative of the difficulty of correctly assaying MP structure and function, most notably being that extensive re-

search over decades still has not led to a consensus on the exact electron transfer mechanism and corresponding electron carrier [55]. A source of the ongoing dispute on cbo_3 functioning is immediately apparent in that even the detergent of choice has a significant impact on the physiology of cbo_3. Assays using the detergent dodecylmaltoside (DDM) or sucrose monolaurate result in cbo_3 co-purified with its natively associated ubiquinone-8 (Q8). However, using the detergent Triton-X does not [56]. This difference is linked to the relatively higher hydrophobic nature of Triton-X compared to other detergents, resulting in stripping of associated lipids and the Q8. The functional significance of this loss is reflected in the lower activity of cbo_3 treated with Triton-X as opposed to the use of DDM; more specifically, Triton-X treatment shows close to zero reduction activity in a flow-flash assay [57].

Achieving physiological relevance in the case of cbo_3 means co-purifying with the relevant electron carriers (quinones) and functional membrane lipids, while maintaining native environment characteristics, part of which can be achieved by preventing excessive and lengthy exposure to detergents. Whereas, if this is not prevented the detergent will strip the MP of lipids and can cause either precipitation or denaturation of the protein, depending on the type of detergent used [17], as is illustrated in Figure 6. Moreover, aiming for physiological relevancy also includes consideration of the structural importance of natively associated ligands. Once more, taking Rumbley *et al* as an example, they report that Q8 is not structurally relevant; they added, ubiquinone-1 (Q1) – a shorter tail quinone with the same headgroup - to a Triton-X-purified cbo_3, lacking Q8, and observed partial restoration of cbo_3 activity. Notwithstanding, neglection of the physiological irrelevance of Q1, assuming it to be a direct replacement for Q8, which is not valid [58]. This is reflected by the inability of Q1 to restore activity completely [57]. Moreover, the true activity of cbo_3 is likely to be much higher than previously assumed, as is indicated in the previously mentioned currently unpublished data [23]. Where, Asseri *et al* added cbo_3 specific native lipids (cardiolipin) which resulted in higher measured activity and elucidated a possible structural function of the ligand/cofactor Q8, reinforcing the notion that the measured activity by Rumbley *et al* might be not fully representative of true activity since the measuring environment is not representative of the native setting. Altogether, it can be argued that not regarding physiological relevance dictating factors, like preservation of native ligands and lipids, dismisses structural validation of the assayed protein. Consequently, invalidating claims like these that dismiss the structural role of natively associated ligands.

Another instance of plainly hidden MP assay uncertainties lays in the seemingly benign assumptions regarding the previously named detergent types, i.e. expecting detergents to not exert influence on MP physiology, which can leave key factors influencing assay outcome unnoticed. For example, Rumbley *et al*, which purified cbo_3 by placing histidine tags on the carboxy-terminus of subunit 2 and applying Ni2+-NTA affinity chromatography, resulting in a sufficiently homogenous isolate, where previous attempts with histidine tags on different subunits resulted in a more heterogenous isolate, which was undesirable since accurate analysis requires a homogenous sample. This apparent structurally differing fraction was regarded as a result of protease activity. Rumbley *et al* assumed a certain native baseline activity of cbo_3 and took that as a reference for whether the protein was denatured. Using this baseline activity assumption, it was argued that purification with DDM did not result in a native-like protein. Conversely,

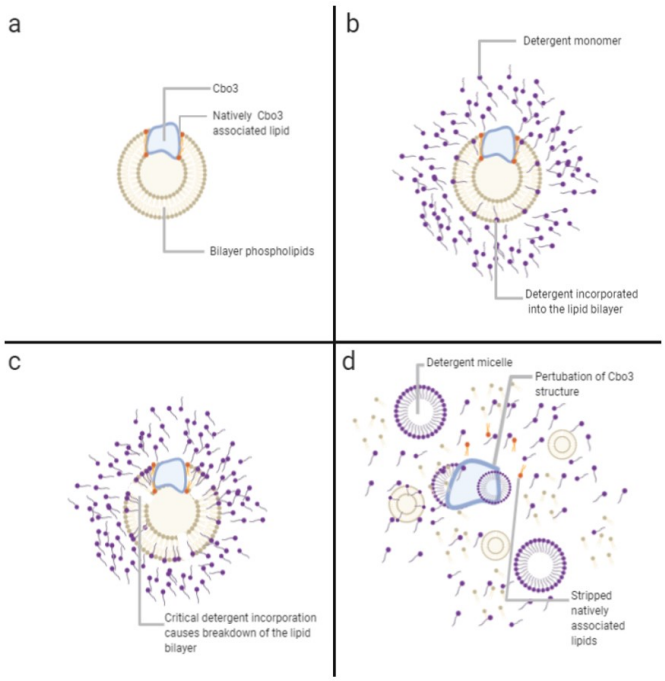


FIGURE 6: Schematic representation of detergent-facilitated extraction of cbo_3. The 4 stages of detergent-facilitated cytochrome bo_3 extraction from the native lipid bilayer. a: Representation of an intact cell with visible cytochrome bo_3 and natively associated lipids. b: The addition of detergent initiates the incorporation of these in the bilayer, a process that is concentration-, time- and temperature-dependent, among other parameters. c: An increasing amount of detergent leads to the collapse of the lipid bilayer. d: The collapse of the lipid bilayer results in the breakage of the components into different structures, loose lipids, detergent monomers, mixed lipid detergent micelles, and micelles with and without the cbo_3 incorporated. The loss of natively associated lipids and cbo_3 structure is indicated. Created using the Biorender web application.

recent unpublished data indicates that this assumed baseline is not resembling the native activity of the protein and that DDM is closer to the native physiological activity than Triton-X [23]. This insight also elucidates the possibility that the undesired heterogenous isolate was an example of the denaturing nature of Triton-X and not necessarily by proteolytic cleavage, as was assumed. It follows that the heterogeneity might be caused by certain fractions being more denatured than others.

The denaturing action of Triton-X-facilitated extraction can also explain the previously unexplained occurrence of heterogeneity in subunit 2 tagged purified samples in the same study. There, it can be argued that the observed heterogeneity, of a fraction failing to incorporate heme into the low spin heme-binding site, is the result of the denaturing effect of Triton-X. Moreover, reduced activity of cbo_3 lacking this incorporated heme groups [56] affirms that his heme group is functionally important and that loss of this group can be seen as a perturbation of protein structure and function. This is a clear example where a natively associated ligand can be attributed with structural and functional importance, and where more careful consideration of the influence of detergent type can result in a different interpretation of assay results.

Another factor that introduces uncertainties in MP assays is the extensive use of washing volumes, like in the case of size exclusion chromatography - for purification purposes. These processes and consequent large washing volumes introduce uncertainties and contribute to the irreproducibility of these assays. For example, Yap *et al* (2006) [59], cbo_3 was suspended in 1% DDM for 2 hours, loaded onto a nickel-nitrilotriacetic acid column, which consisted of 3 times 25 ml bed vol 0.1% Triton-X followed by 3 times 25 ml high salt wash with 0.1% Triton-X, with more bed volumes until no color was perceived, purified with 1% triton and 1.25% OG for 1 hour, and then dialyzed overnight.

6 Assay platforms

As described in the detergent section, when a membrane protein is in solution detergent micelles are present alongside protein-detergent complexes (PDC's). PDC's are the most common format in which membrane protein assay activity is described, yet totally lack the physiological characteristics that the native bilayer provides. Membrane-based assays offer an alternative platform with more native-like qualities, where it has been shown that probing a protein like cbo_3 in either a membrane or solution-based assay directly influences electron transfer rates. In general, the solution based assays achieve higher levels of activity. However, if one is trying to study biology, then the physiological relevance of the solution-based assay, or lack thereof, needs to be taken into account. We continue in this section with cytochrome bo_3 as a core theme because it utilizes reduced membrane-bound substrates (quinones) along with molecular oxygen. This naturally allows the use of a multitude of approaches that can be taken to study it. Cytochrome bo_3 is also a perfect system to explore the role of the membrane, which actively translocates protons - informing us of membrane integrity. Moreover, the E. coli lipids used in said essays are intensively studied and well described.

6.1 Solution-based assays for membrane proteins

Below we described some of the most used and relevant downstream assay types for a solution-based assay of membrane proteins, with cbo_3 being a versatile case study model protein, these methods probe the activity of the protein of interest by measuring differences in certain reaction substances or parameters, like differences in oxygen or pH-responsive fluorescence of ACMA. The point of attention lays in the effect the assay type has on the measured outcome, the influence of the used detergents on the assay, and the physiological relevance of said assays.

Analysis of oxygen consumption as a consequence of cbo_3 activity allows probing of the influence of the factors influencing said cbo_3 activity. An often-used method to probe the oxygen consumption is the Clark-type electrode – an electrode that can measure the soluble oxygen concentration by reduction of oxygen into water, which requires adding an electron donor and reduced substrate to the detergent incubated sample in the electrode chamber, which jumps-starts oxygen consumption from the aerated electrode. Points of consideration for the physiological relevance of this assay lay in the physiological relevance of the added substrates, such as the short tail quinones, or of the previously discussed effect of the present and previously applied detergents, such as DDM. Examples of this kind of application are probing of the effects of the carboxy-terminal insert in the Q-loop of *Escherichia coli* cytochrome bd-I [60] or measuring the anti-mycobacterial activity of cytochrome bcc inhibitor Q203 and the influence of small-molecule inhibition of cytochrome bd [61].

Another method for the probing of protein activity is the measuring of fluorescence quenching of pH-responsive fluorophores 9-amino-6-chloro-2-methoxyacridine (ACMA), which has quenched fluorescence as a consequence of the pH changing due to protein activity. An example of this being applied is in the study of ionophoric effects of the antitubercular drug bedaquiline (BDQ), specifically using cbo_3 proteoliposomes [62]. For ACMA quenching the same considerations regarding added substrates and used

detergents apply.

6.2 Membrane-based assays for membrane proteins

Membrane-based assays offer an alternative to solution-based assaying of protein structure-function, which differs in that the protein of interest is reconstituted into a membrane structure, offering more native-like qualities. Two often used examples of such a membrane-based MP assay are the tethered bilayer lipid membrane (tBLM) and single-molecule bioelectrochemistry assays.

Reconstitution using self-assembled monolayers (SAMs) into a tethered bilayer lipid membrane (tBLM) is an effective tool for structure-function MP research. However low efficiency of surface reconstitution and lack of control over the enzyme orientation at assembly on the electrode-solution interface remain problems limiting application [63]. Figure 7 is an illustration of a general MP reconstitution into a tBLM using SAMS. An example of a study using tBLM is the activity assay of the respiratory-chain enzyme CymA, which probed the effect of the presence of its native redox partner flavocytochrome c3 (Fcc3) fumarate reductase [64]. In this instance, tBLM was used since CymA requires a membrane to form long-lasting respiratory complexes. In solution-phase assays, transient complexes are observed in vivo. Whereas, in an electrochemical assay platform the complexes are long-lasting.

Single-molecule bioelectrochemistry assays also make use of SAMs on a gold electrode, however now the protein of interest is incorporated into proteoliposomes, which, due to the low protein-to-lipid ratio, only sparsely incorporate the protein. Therefore, allowing single molecules to be probed. An example of this assay type being the previously mentioned assay measuring the proton flux of cbo_3 [7].

6.3 Solvent-free MP analysis using Mass spectrometry

Mass spectrometry of MPs incorporated into amphipols, bicelles, and nanodisks offers a detergent free alternative for structural analysis. With the functionality to provide insights into stoichiometry and lipid interactions. Thereby offering insights which other conventional detergent alternatives are unable to reliably provide [65].

6.4 Assay platform considerations

Regarding the choice of assay platform, it is not only beneficial to use the best-suited assay platform for the protein structure-function of interest but the detergent of choice is once more a parameter of importance. This difference is reflected in the opposing findings for the proposed electron transfer dynamics of *Escherichia coli* NDH-2, by either Godoy-Hernandez *et al*, who used a tBLM (membrane-based assay), or Nakatani *et al*, who used a solution-based assay and using detergents to solubilize the NDH-2 [21],[66].

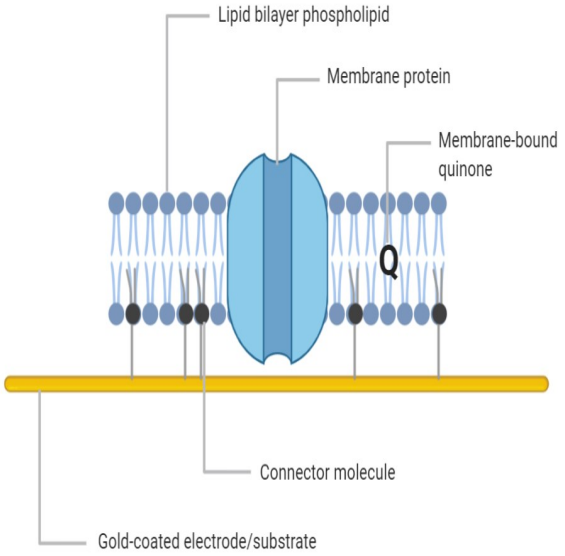


FIGURE 7: Illustration of a SAM assay, specifically a tethered bilayer membrane system (tBLM) with a reconstituted MP. Here the lipid bilayer with incorporated membrane protein is attached to the gold-coated electrode/substrate trough connector molecules, added membrane-bound quinones allowing charge transportation in electrochemistry experiments using this assay. Created using the Biorender web application.

These distinct assay types tend to introduce differences between assays and the context in which their results can be interpreted. For instance, biochemical conditions for crystallization or NMR in terms of buffers and especially detergents are significantly different from those required for a functional protein assay. For either crystallization or NMR, the more homogenous the sample, the better the resolution. This is achieved by using larger columns, consequently exposing the protein to a larger volume of buffer and detergent. Conversely, for activity-based studies, this is not a feasible method, since most times one cannot extensively apply these detergents without causing a loss of function [17], which is consequently reflected by decreased activity of the protein of interest. The differences in sample requirements mean that the assay parameters, such as detergent type and detergent exposure time, which might be suited for one type of assay will probably not translate to the other. It can be argued that the extensive use of detergents normally applied in a crystallography assay might not even be justified, since extensive washing changes the structure

and state of a protein to such an extent that the assay can often not be considered to be physiologically relevant. Even though it might be well suited for high yield structural analysis, one can argue that since it does not represent the native protein physiology; then its structure might also not be representative. For NMR this difference is even more pronounced, reflected by the fact that the most used detergent for said assay is an example of a previously mentioned harsh detergent, n-Dodecylphosphocholine - also known as Fos-Choline-12 (FC12). Within the line of reasoning introduced previously, FC12 is a harsh detergent that is well suited for creating homogenous samples, leading to a high yield. However, this is at the cost of possible potential perturbations of the native protein structure and function [67]. Chipot *et al* observed and verified that detergents like FC12 are significantly denaturing and therefore negatively contributing to a physiologically relevant MP assay, which was reflected in the often lower activity observed when using these detergents compared to using more mild variants [67]. Moreover, probing of the effect of FC12 on mitochondrial carriers by Kurauskas *et al* further reinforces the notion that FC12 facilitated assays do not result in the mapping of functionally active proteins [68]. Therefore, where the denaturing effect of certain detergents is already taken into account with functional studies progress is to be made in doing the same in crystallographic and NMR assays. However, both structural and functional assays could benefit from increased scrutiny on the extent to which extended and excessive exposure to detergent is necessary and desired.

7 Outlook

Despite the currently limited depth of literature regarding the influence of parameters, such as detergent type/concentration, temperature, and exposure time, there seems to be a movement towards a more fundamental approach to understanding the different facets of MP assays. Moreover, the scarcity of literature covering this topic also provides ample opportunities for relatively simple research to greatly improve our understanding, which could greatly benefit the field of membrane biochemistry in seeking and obtaining more physiologically relevant assays. Whether that be in the form of alternative extraction methods that have more native qualities or being more conscious and proactive in protecting native structure when performing assays [69], [70]. Most notably assays using detergents are becoming more refined [71]. The latter by using milder more structural supportive detergents such as LMNG and taking into account more parameters such as temperature and detergents concentration/exposure time. However, big strides can still be made. Mentioned examples being the taking into account of the T_m of the native environment of an MP, or measuring the detergent concentration in different steps of the assay, among other considerations.

8 Conclusion

Current methods for assaying of MPs require extraction and purification. And, until alternative extraction methods become more economically and technically viable, detergents will continue to be the main tool used for doing so. What could be improved upon is the awareness and consequent assaying of unaccounted for factors influencing assay outcome, with an increased emphasis on the physiological relevance of an assay. Does an extensively detergent washed protein actually represent native structure and function? Moreover, how do detergent -type, concentration, and exposure time influence assay outcome? To what extend is a perturbation of native protein structure reflected in the loss of natively associated lipids and ligands? Do detergents themselves engage in a structural role when applied to MPs? This review elucidated several examples where seemingly apparent uncertainties where not being addressed. Specifically, the influence of the combined T_m of different components, the physiological relevance of natively associated lipids/ligands, the application of harsh detergents like Fos-Choline-12 in structural studies, the influence of excessive exposure to detergents trough large washing volumes, the beads used in both extraction/purification and changes in detergent concentration during the assay. In summary, all these efforts seek to resolve uncertainties regarding an assay and enhance physiological relevancy by preserving native structure - most critically native lipids and ligands. To achieve such a feat requires further research into the nature and dynamics of the native bilayer combined with research on the implications of current methods on the physiological relevance of an MP assay. Besides seeking a more physiologically relevant assay, new alternatives for detergents could offer solutions in the future. For now, detergents are and will continue to be a key tool in MP assaying, however their use and extend of application could be more carefully reflected upon.

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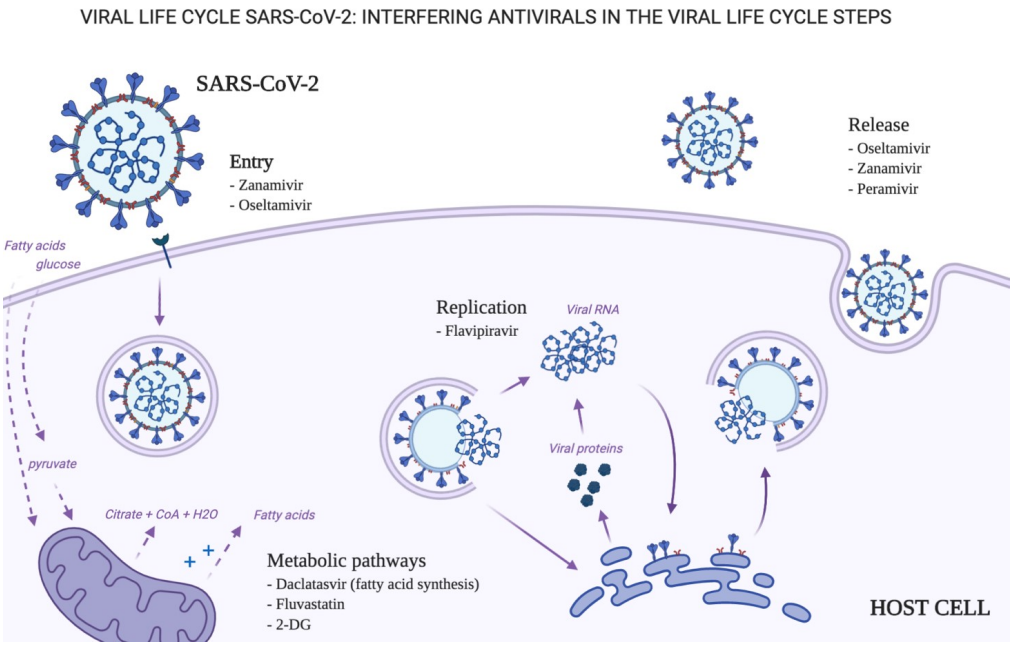
Targeting Steps in the Viral Life Cycle of SARS-CoV-2: a Review of the Insights of the Current Antiviral Strategies of Influenza A Potentially Applicable to SARS-CoV-2

Hannah Hulsewé Polina Kostina Flip Jansen Lucas Hofmans Anoek Huijsmans Rochelle Niemeijer

Graphical Abstract



IN BRIEF

An extended description of the viral life cycle parts of Influenza A and SARS-CoV-2 is elucidated in depth. Additionally, the existing antivirals strategies for Influenza A are outlined and its potential applicability to SARS-CoV-2 is discussed.

HIGHLIGHTS

- Mechanism of the viral life cycle parts of Influenza A
- Mechanism of the viral life cycle parts of SARS-CoV-2
- Antiviral approaches of Influenza A can provide anticipation of new developments for new pandemics

Corona Super Research Project, TU Delft, Erasmus MC, July 2020

Targeting Steps in the Viral Life Cycle of SARS-CoV-2: a Review of the Insights of the Current Antiviral Strategies of Influenza A Potentially Applicable to SARS-CoV-2

Hannah Hulsewé Polina Kostina Flip Jansen Lucas Hofmans Anoek Huijsmans Rochelle Niemeijer

Abstract

The pandemic in 2019 caused by the zoonosis of SARS-CoV-2 showed that the world is currently insufficiently prepared for properly containing outbreaks, highlighting a need for rapid antiviral discovery. Recent advancements in the fields of proteomics, metabolomics and metagenomics among others have enabled more precise measurements that allow the structures and dynamics of the viral life cycle to be mapped in much greater detail. During this developmental period, new antiviral targets have been identified. In order to establish attractive potential antiviral targets for SARS-CoV-2, the interference points in the viral life cycle of the pathologically relevant Influenza A virus (IAV) have been reviewed. This review provides in-depth insight into the modes of entry, replication, release and influence on the host cell metabolic pathway of SARS-CoV-2 and IAV. This review of different points in the viral life cycle has elucidated points of interference and corresponding potential antivirals. Further research into viral life cycle interference points is a crucial part of a structural framework enabling more rapid antiviral discovery. As the given viruses have similarities, the knowledge of IAV can contribute to the rapid development of antivirals for SARS-CoV-2. This research emphasizes the importance of experiences gained by extensive research on IAV. This allows the development of a roadmap towards more rapid antiviral discovery with applications for future viral outbreaks.

Keywords: SARS-CoV-2, COVID-19 Pandemic, Influenza A, viral life cycle, antivirals

1 Introduction

In December 2019, an outbreak of a novel coronavirus occurred in Wuhan City, China. The viral RNA sequence was published on the 7th of January. The virus was later named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causing the disease COVID-19 (1) As of June 25th, over 9 milion cases have been confirmed worldwide (2). The estimated mortality rate is 7%. Like SARS-CoV and MERS-CoV (Middle East Respiratory Syndrome-virus), SARS-CoV-2 is a betacoronavirus with a single strand positive sense RNA genome (3).

This report will compare SARS-CoV-2 to Influenza A virus (IAV) and try to apply the experience with the Influenza virus to SARS-CoV-2. Several influenza strains have caused pandemics, for example the Spanish flu of 1918 and the swine flu of 2009, which are both H1N1IAVs. Furthermore, both SARS-CoV-2 and IAV are respiratory viruses that spread through droplets (3). In this report the focus will be on the viral life cycle and at which points in the viral life cycle antivirals can intervene. The viral life cycle consists of entry, replication, assembly and release. The characteristics of both virions and their respective viral life cycles are explained below. Figure 1 depicts a general summary of these stages. The mechanisms of the specific steps in the viral life cycle will be elaborated in various sections of this report.

1.1 SARS-CoV-2

The viral RNA of SARS-CoV-2 encodes multiple proteins, of which four are structural (3). The virion comprises a helical nucleocapsid (N) in which the viral genome is encapsidated. Around the nucleocapsid there is a viral membrane containing three glycoproteins: spike (S), envelope (E) and membrane (M) (4).

The entry of SARS-CoV-2 is enabled by the binding of the S-protein to its receptor angiotensin-converting enzyme 2 (ACE2) and Transmembrane protease serine 2 (TMPRSS2) on the cell surface. The viral RNA enters the host cell's cytoplasm either through direct fusion with the plasma membrane or through endocytosis, followed by fusion with the endosome. The RNA of SARS-CoV-2 encodes for two polyproteins, pp1a and pp1ab, which are cleaved into 16 non-structural proteins (nsp), like RNA-dependent RNA-polymerase (RdRp). These non-structural proteins replicate the viral RNA and produce subgenomic mRNA that

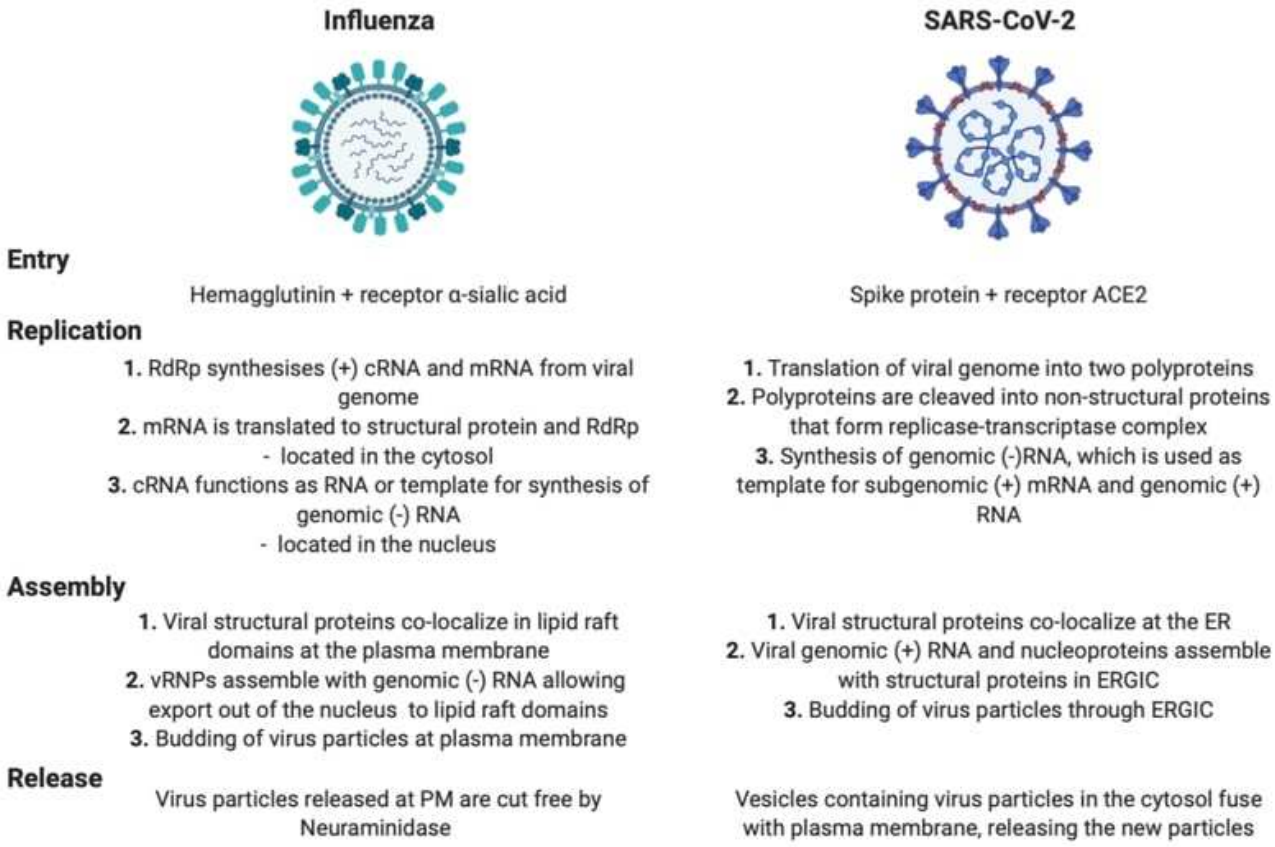


FIGURE 1: An overview of the viral life cycles of Influenza and SARS-CoV2

encodes for structural and accessory proteins. Assembly of the SARS-CoV-2 virion takes place in the rough endoplasmic reticulum (RER) and Golgi apparatus. The particle then buds through the ER-Golgi intermediate compartment (ERGIC), after which the virion is enclosed in a vesicle through a process called budding. The virus is released from the cell when the vesicle fuses with the plasma membrane (figure 2) (4).

1.2 Influenza

Influenza is an enveloped negative stranded RNA virus. The influenza virion has three main components: the core (viral ribonucleocapsid [vRNP]), the matrix protein (M1) and the viral envelope. The viral envelope surrounds the vRNP and is made up of a lipid bilayer with M1 on the interior and spikes composed of the viral glycoproteins haemagluttinin (HA), neuraminidase (NA), and matrix-2 (M2) on the exterior (6).

The entry of influenza A viruses is initiated by binding of HA to salic acid, which induces endocytosis. The viral RNA is released through fusion of the viral membrane with the endosome and the viral genome enters the nucleus subsequently (7).

Since influenza is a negative stranded RNA virus, the RNA is transcribed to form a complementary positive stranded RNA (cRNA), using the RdRp bound to the viral RNA. This cRNA can be used as a template for new negative stranded genomic RNA or it can be translated to form proteins. The RNA replication takes place inside the nucleus, as opposed to SARS-CoV-2 which replicates in the cytoplasm (8). The RNA of influenza is associated with proteins to form viral ribonucleoproteins (vRNPs) and travel through the nuclear pore complex into the cytosol. Virion assembly is localized at lipid raft domains in the plasma membrane. Release of Influenza takes place through budding, which causes an envelope to cover the virion (9). This process is depicted in figure 3.

1.3 Antivirals

Due to the severity of the current SARS-CoV-2 outbreak and its high mortality rate, it is essential that a treatment for COVID-19 is found. Antivirals are compounds that inhibit viral development. This can be achieved by enhancing the immune system or by interfering in specific steps in the viral life cycle (11). This report will focus on antivirals that interfere at entry, replication, or release and antivirals that

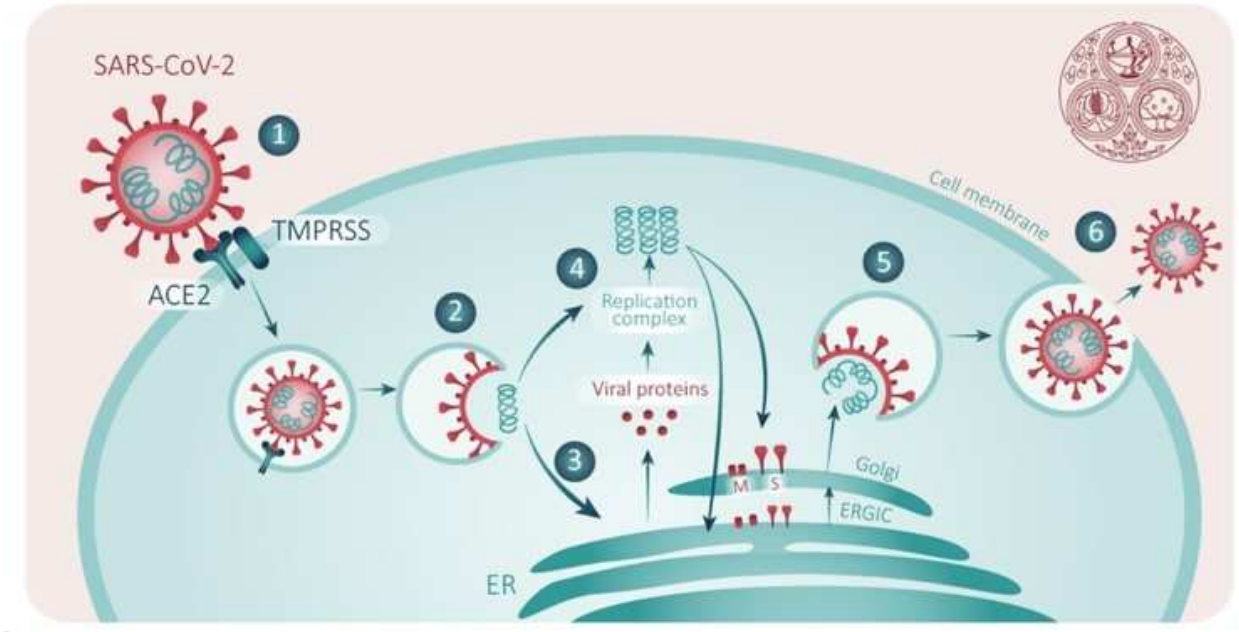


FIGURE 2: (5): A schematic overview of the viral lifecycle of SARS-CoV-2. 1: The Spike protein of SARS-CoV-2 binds ACE2 and TMPRSS, which causes endocytosis. 2: The virion fuses with the endosome, releasing the viral genome. 3-4: This encodes for two polyproteins that are cleaved and form a replicase-transcriptase complex. 5: The virion assembles at the ERGIC. 6: It is eventually released from the cell when it fuses with the plasma membrane

interfere in the viral metabolic pathways (12).

The Corona Super Project organised by the TU Delft and Erasmus MC aims to develop strategies to combat the current SARS-CoV-2 pandemic. This report explains the viral life cycle of SARS-CoV-2 step by step and compares it to Influenza A. Furthermore, the interference points of antiviral treatments are pointed out. The goal is to determine which antivirals for Influenza A can be used to treat SARS-CoV-2. This report gives an overview of potentially effective treatments against SARS-CoV-2 and can serve as a starting point for understanding SARS-CoV-2 by comparing it to the well-studied Influenza A virus.

Influenza A has many similarities to SARS-CoV-2, for example the transmission through aerosol droplets, the pandemic potential and symptoms such as cough or fever (3). While, SARS-CoV-2 is a newly emerged virus, IAVs has been circulating for many years now. Currently, research is being done into the application of several antivirals, including those used in treatment of influenza, in the current SARS-CoV-2 pandemic. In this report, a selection of antivirals against IAV, as well as their intervenance points and their applicability for SARS-CoV-2, is elaborated. The findings outlined in this report are summarized in table 1 (Appendix A) and all the listed antivirals in this table correspond to the graphical abstract where it can be seen at which point in the viral life cycle they interfere.

2 Entry

2.1 Influenza Entry

HA and NA are distributed on the surface of influenza virus particles. Like S protein of coronaviruses, HA and NA are important targets of antiviral drugs and vaccines. IAVs initiate the infection process by binding HA molecules to surface glycoconjugates containing terminal SA (Ser, Ala) residues with the HA receptor-binding site. The IAVa scans the cell surface for the proper sialylated “receptor” by using the sialidase function of NA to remove local SAs and liberate non-productive HA associations (13), then the envelope protein haemagglutinin binds α-2,6 - or α-2,3 -linked 2 sialic acid as host cell receptor (14). Despite the unknown identity of the receptor, it is clear that HA-mediated binding to the receptor triggers endocytosis of the virion. Endocytosis can either occur in a clathrin-dependent manner, involving dynamin and the adaptor protein Epsin-1, or by macropinocytosis. Once inside the cell, the virus is trafficked to the endosome, where the low pH activates the M2 ion channel. Opening of the M2 ion channel acidifies the inside of the viral particle, releasing the packaged vRNPs from M1. The low endosomal pH also causes a large conformational change in HA that exposes the fusion peptide, enabling transfer of the vRNPs to the host cytoplasm (13).

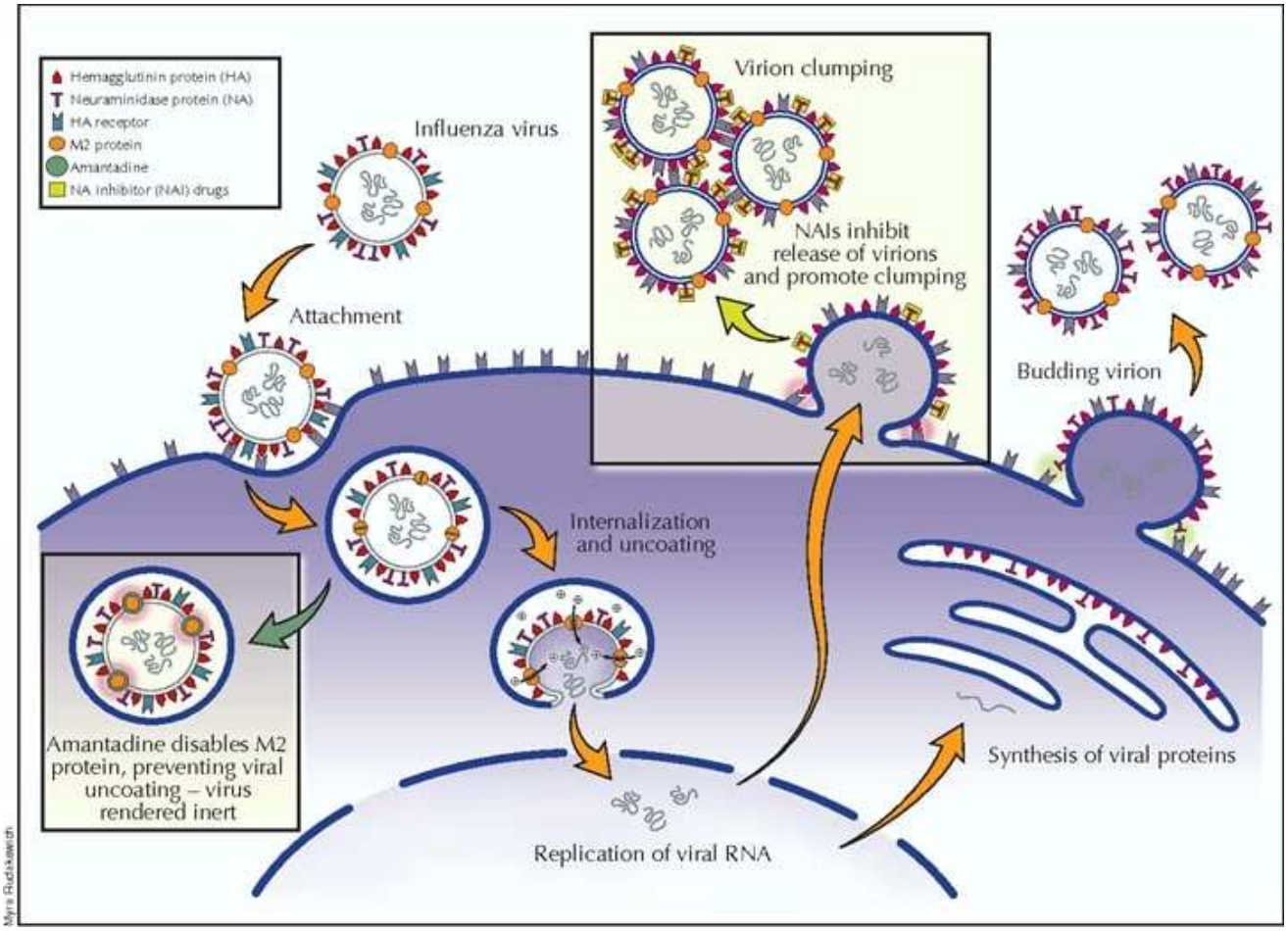


FIGURE 3: (10): A schematic representation of Influenza A virus life cycle: attachment, entry, replication and release. Hemagglutinin binds the receptor sialic acid. This leads to endocytosis. The virion fuses with the endosome and releases the viral genome. RdRp synthesises cRNA and mRNA in the nucleus. mRNA is translated in the cytosol. The virion assembles at lipid raft domains in the plasma membrane, where it leaves the cell through budding.

2.2 SARS-CoV-2 Entry

Coronavirus entry is mediated by surface-anchored S protein, which is present as a trimer with three receptor-binding S1 heads on top of a trimeric membrane fusion S2 stalk. SARS-CoV S1 subunit is responsible for receptor binding, it contains a receptor-binding domain (RBD) that recognizes ACE2. The RBD constantly switches between perfusion and postfusion conformations; a lying-down position for immune evasion and a standing-up position for receptor binding respectively. S2 subunit forms the stalk-like region of the spike and it is needed for membrane fusion, for which proteolytic activation of spike is required. So at the S1/S2 boundary S1 dissociates and S2 undergoes a significant change in its structure. These SARS-CoV entry-activating proteases include cell surface protease TMPRSS2 and lysosomal proteases cathepsins (15).

IAV HA is prone to proteolysis during the virus packing, coronavirus S proteolysis occurs later, during the cell entry process or after receptor binding. Proteolysis of S1 results in direct membrane fusion, so it is an essential trigger for membrane fusion. There are four distinct stages of the virus infection cycle, when the host proteases can cleave the coronavirus spikes:

1. Virus packaging in virus-producing cells - proprotein convertases (e.g., furin);
2. After the release of the virus into extracellular space - extracellular proteases (e.g., elastase);
3. After virus attachment to virus-targeting cells - cell surface proteases (e.g., type II transmembrane serine protease (TMPRSS2));
4. After virus endocytosis in virus-targeting cells - lysosomal proteases (e.g., cathepsin L and cathepsin B).

Not only proteolysis, but additional triggers/factors can take place, it could be low pH or receptor binding, which also affects membrane fusion (16).

SARS-CoV entry is less efficient than SARS-CoV-2 entry. The cell entry of SARS-CoV-2 is activated by the protein convertase furin, which reduces the dependence on target cell proteases for entry. SARS-CoV-2 maintains its efficient cell entry because the binding with the RBD has a high affinity (17).

2.3 Antivirals

Zanamivir and oseltamivir, which both are neuraminidase inhibitors (NAIs) that are used in treatment of both SARS-CoV and IAV. However, there is no exact evidence that NAIs are helpful during the SARS-CoV-2 treatment, although they were effective for MERS-CoV (13, 18-20). The NAI drugs, zanamivir and oseltamivir, bind to the active site on the viral neuraminidase, blocking its activity. Thus, virus particles cannot exit the cells as easily, and they tend to clump and not disperse. This impedes their ability to infect more cells and attenuates the patient's infection (1). Detailed information about these two antivirals you can find in the "Release" section.

The antiviral effect of Arbidol has been observed in vitro via testing CPE (Cytopathic effect) of infected cells, and it showed that this antiviral inhibits the entry process (75%) and the post-entry stage (55% inhibition rate) - quantified by qRT-PCR, then the result was confirmed by western blot and immunofluorescence microscopy (15). CPE defines changes of cell structure after the invasion of a virus, so this antiviral can be considered as a good candidate for COVID-2019 treatment. Arbidol (Umifenovir) targets viral HA, blocking the virus entry into the cell (membrane fusion and endosome formation). Importantly, this antiviral drug is not approved by the US FDA (20).

It is not excluded that zanamivir and oseltamivir do not inhibit the entry mechanism of SARS-CoV-2. As MERS-CoV is sensitive for this drug inhibiting at the entry step it can be expected that SARS-CoV-2, containing similar membrane proteins which are essential for the entry part, will be sensitive for this drug as well. In contrast to arbidol, which is an Influenza A drug that is an efficient inhibitor of SARS-CoV-2 in the entry part.

3 Replication

3.1 Replication Influenza

The replication cycle of influenza viruses, unlike many other RNA viruses, takes place in the nucleus of the host cell.

The vRNPs of IAV are composed of negative sense RNA, RdRp and NP. Influenza RdRp consists of three polypeptides: polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acidic (PA). PB1 is the polymerase active site and PB2 and PA together are required for transcription initiation via cap-snatching. PB2 houses the cap-binding domain and PA is responsible for the endonuclease activity. Cap snatching is a process in which the 5' cap of host mRNA is cleaved by PA and used as a primer for viral mRNA synthesis (21, 22).

The vRNAs bind the C-terminal domain (CTD) of host DNA-dependent RNA polymerase II. A phosphorylation on serine-5 of the CTD is needed for initiating polymerase II and is recognized by RdRp. RdRp binds to pre-mRNA at 10 to 15 nucleotides from the cap with the PB2 subunit and cleaves it using the PA endonuclease domain. RdRp then aligns the bases of the vRNA and the cap structure and elongates it using the vRNA as a template until it reaches an oligo-U tract. Here the RdRp stutters, creating a poly-A-tail on the viral mRNA. This results in the production of a viral capped and polyadenylated mRNA that is transported out of the nucleus and translated in the cytosol (13, 22, 23).

Translation of viral mRNA results in the production of new viral proteins, including RdRp. For replication, a complimentary copy of the vRNA is produced. These complementary RNAs (cRNA) are bound by RdRp in the active site of the PB1 subunit. The cRNAs bind to newly synthesised NP and one RdRp copy when exiting the polymerase to form complementary RNP (cRNP) complexes. These complexes synthesize new uncapped vRNA that associates with NP and RdRp to form vRNPs (13, 22).

3.2 Replication SARS-CoV-2

As mentioned before, the viral positive sense RNA encodes two polyproteins; pp1a and pp1ab and is translated directly upon entry into the cytosol. The polyproteins encode for non-structural proteins and are cleaved by intrinsic pappain-like proteases encoded in nsp3 and nsp5. Cleavage of polyproteins leads to the production of 16 nsps that are involved in numerous viral and host processes, like host defence evasion and viral replication. Many nsps assemble into the replicase-transcriptase complex. Nsp12 is the RdRp, which uses nsp7 and nsp8 as cofactors. Nsp13 is an RNA helicase and nsp14 has an exoribonuclease domain that allows for proofreading and post-replicative RNA repair, a feature unique to coronaviruses. The replicase-transcriptase complex transports into intracellular double-membrane vesicles derived from the RER and rearranged by nsps. Here RdRp produces negative sense subgenomic and genomic RNAs. These serve as templates for the production mRNAs and positive sense genomic RNA respectively (24-26).

3.3 Treatment

Viral genome replication and transcription require host proteins. Therefore, it is important that antivirals target virus specific proteins in these processes to prevent adverse effects on the cell's own transcription and translation processes. For RNA-viruses, these drugs often target the RdRp. Drugs that

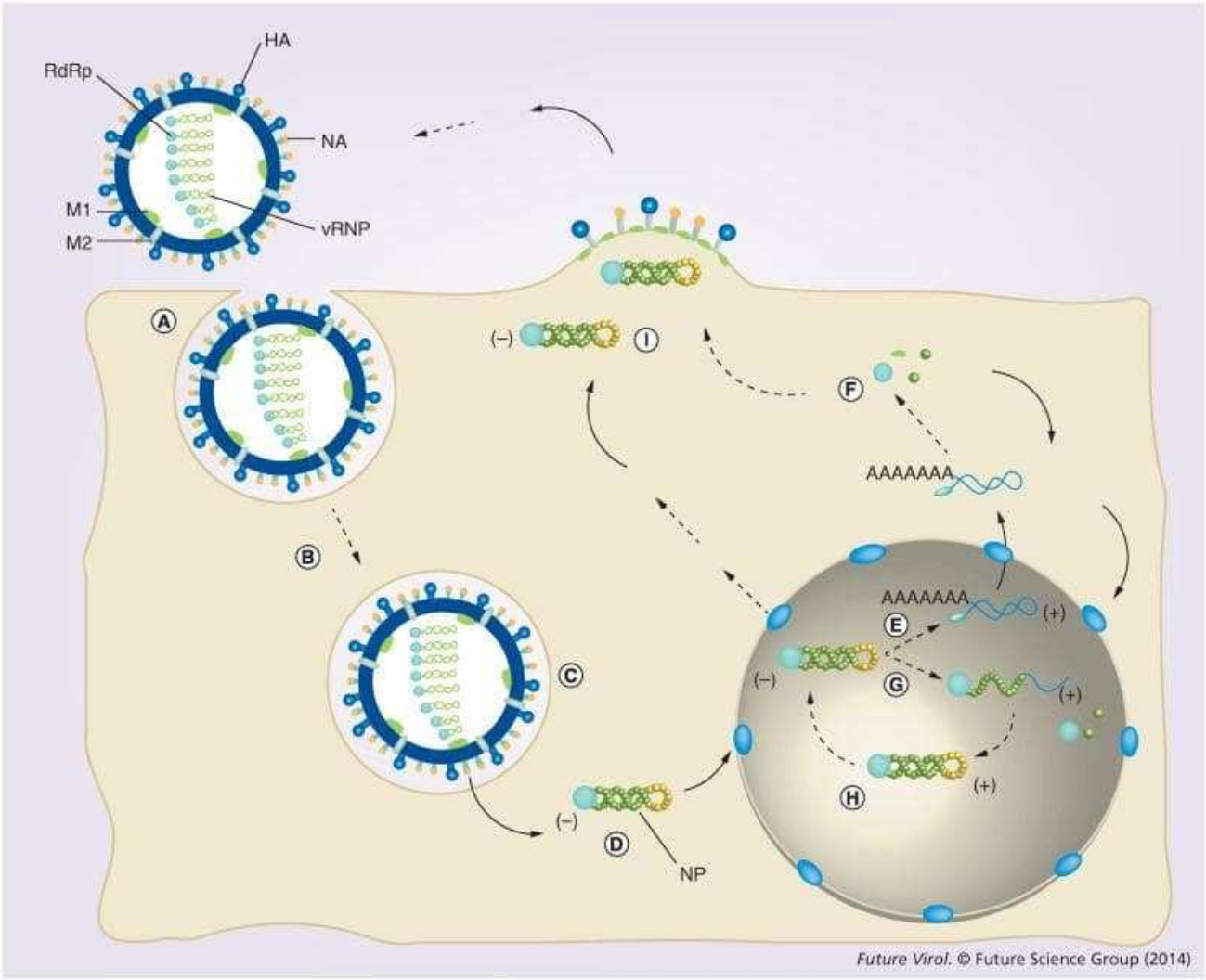


FIGURE 4: (22): Schematic overview of Influenza A replication. The vRNPs are transported into the nucleus. Here viral capped and polyadenylated positive sense (+) mRNA's are produced, as well as a (+) template for the synthesis of new genomic negative sense (-) RNA.

target influenza RdRp are pimodivir, baloxavir marboxil, and favipiravir. Pimodivir and baloxavir marboxil both inhibit the cap-snatching process needed to initiate viral mRNA synthesis. Pimodivir targets the PB2 subunit, preventing binding to the host cap structure, whereas baloxavir marboxil inhibits the cap-dependent endonuclease activity of the PA subunit. Favipiravir, or T-705, is a purine analog and is used as an alternative substrate by RdRp. Both pimodivir and baloxivir marboxil are specific for influenza viruses, since they target cap-snatching. Because of targeting an influenza specific process, application of these drugs to other viruses is limited (27). In contrast, favipiravir is a broad-spectrum anti-RNA virus drug that has antiviral effects against multiple RNA viruses, including respiratory syncytial virus (RSV), poliovirus and influenza A, B and C. It was also used during the 2014 Ebola epidemic in West Africa (23). Since its broad specificity it has quickly become of interest during the SARS-CoV-2 outbreak.

3.3.1 Favipiravir in Influenza

Favipiravir is mainly acting as a purine analogue and results in mutations in viral RNA results due to errors during viral RNA synthesis (27). Favipiravir is approved in Japan for treatment of non-seasonal influenza viruses that are resistant to other antivirals. Its efficacy was studied in animal models. Mice were infected with influenza A and got oral administration of favipiravir or oseltamivir, a widely used influenza treatment. In lethal and nonlethal influenza infection models, the efficacy of favipiravir and oseltamivir was compared. In lethal influenza infections, favipiravir proved to be more effective than oseltamivir but in nonlethal models

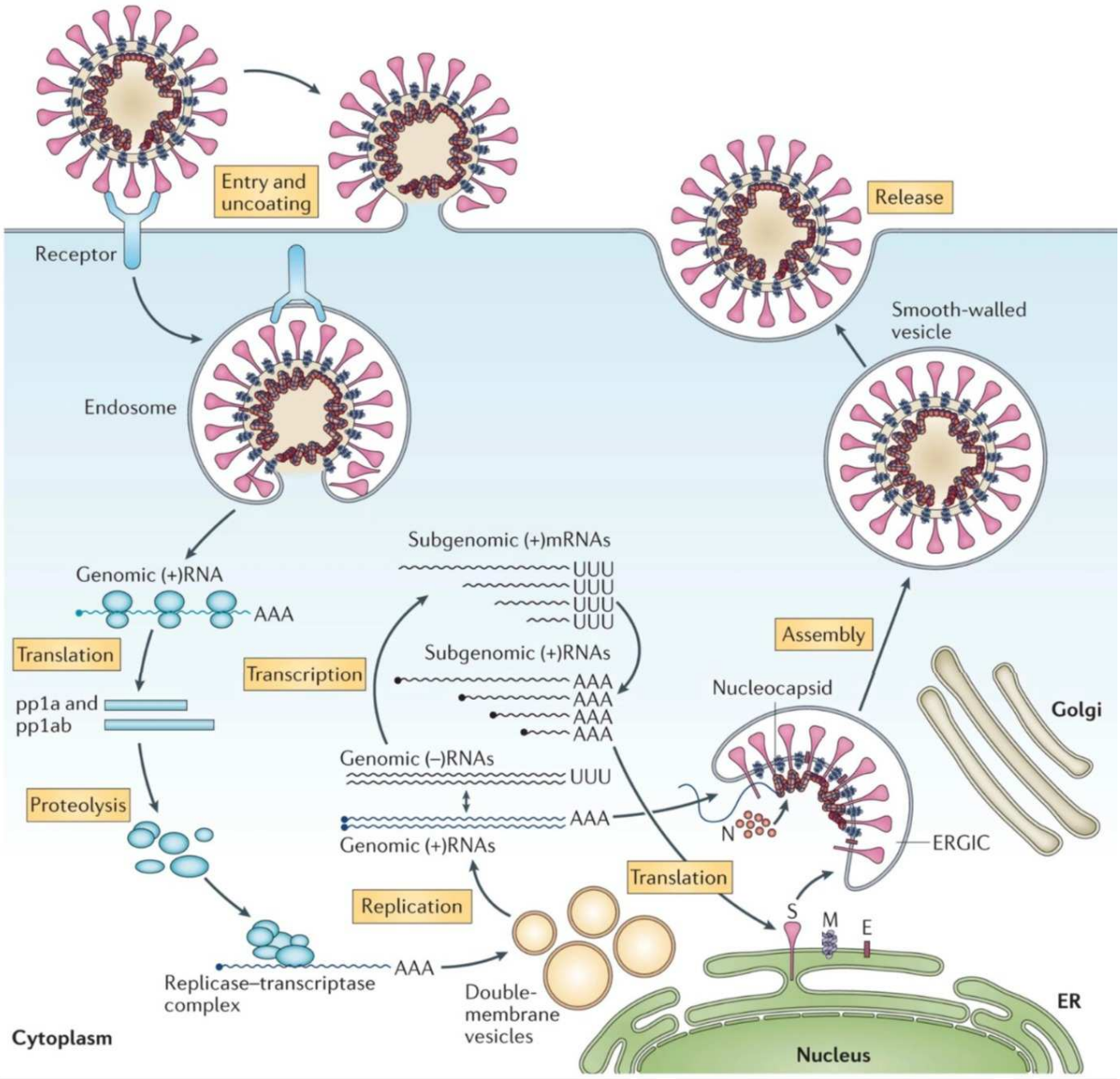


FIGURE 5: (24): Schematic representation of SARS-CoV-2 replication. The genomic positive sense (+) RNA is translated into pp1a and pp1ab which are cleaved. Products then form the replicase-transcriptase complex. This facilitates the production of subgenomic mRNA

the antiviral effects were comparable (23). It was also shown that it is specific for RdRp and does not affect the synthesis of cellular DNA or RNA and has no effect on DNA viruses (28, 29).

Faviripiravir is a prodrug that becomes active through phosphorylation by purine phosphoribosyltransferases. By this phosphorylation, favipiravir ribofuranosyl-5'-triphosphate (favipiravir-RTP) is formed that can function as a substrate for RdRp (27, 29). The mechanism of action (MoA) of favipiravir has not been fully elucidated. Sequence analysis showed that favipiravir led to an increased mutation frequency causing lethal mutagenesis (30). Furthermore, it was shown that favipiravir functions as a chain terminator for viral RNA synthesis, blocking strand extension. Because it inhibits the production of functional genomic RNA, the formation of genomic RNA containing drug resistant mutations is prevented (29).

3.3.2 Favipiravir in SARS-CoV-2

As mentioned before, favipiravir hinders viral replication via lethal mutagenesis or chain termination. SARS-CoV-2, as well as other coronaviruses, has nsp14 which has an exonuclease activity allowing post-replicative RNA repair. This can lead to a reduced antiviral effect of nucleotide analogues by removing them. Nevertheless, after favipiravir treatment of cells infected with SARS-CoV-2, sequence analysis of the viral RNA showed a 3-fold increase in mutation frequency. This may be due to the fact that SARS-CoV-2 genome has a low cytosine content and favipiravir puts more strain on the unbalanced, purine rich nucleotide content. Nsp12 functions in a primer-dependent way and the MoA of favipiravir in the SARS-CoV-2 infection was studied using annealed primer template (PT) and selfpriming hairpin (HP) RNA. This priming may result in an increased stability of the elongation complex. These studies revealed that there are different MoAs for favipiravir in SARS-CoV-2 infection. When PT was used, the MoA was dependent on whether incorporation was instead of adenosine or guanine. When favipiravir replaced adenosine, extension of the RNA products was inefficient and when it replaced guanine elongation is rapid but the complex stalls before incorporation of the analog. In the HP model, the analogues were efficiently incorporated and suggesting lethal mutagenesis as the MoA. These studies suggest that in SARS-CoV-2, the antiviral effect of favipiravir is due to a combination of chain termination, slowed RNA synthesis and lethal mutagenesis (31). A randomized clinical trial by Chen et al. (2020) compared the effects of favipiravir treatment with arbidol, a widely used influenza antiviral targeting HA. These studies showed that favipiravir treatment in patients without diabetes or hypertension had a higher recovery rate at the end of the 7 day treatment, as well as a shorter time for cough relief and fever reduction (32).

4 Release

4.1 Influenza Release

Influenza viruses use lipid raft domains in the plasma membrane of infected cells for viral assembly and budding. Viral budding is a method a virus uses to leave the infected cell, thus releasing the viral progeny particles. In this step, finalizing viral assembly, the viral envelope is obtained from the infected cell's plasma membrane. Lipid raft domains are cholesterol- and sphingolipid-enriched regions of the plasma membrane. HA protein can cause a coalescence of lipid raft domains, forming a so-called viral 'budozone'. Consequently, HA and NA are associated with lipid raft domains (9). Furthermore, HA and the M1 protein are involved in completing viral budding. Also, it has been postulated that M1 crosslinks the cytoplasmic tails of HA and NA, mediating incorporation into the budding virion. In contrast to a number of other viruses, which need the proteins of the endosomal sorting complexes to transport ESCRT complexes, membrane scission to release the viral progeny particles requires the M2 protein. The NA protein removes sialic acids from host cells and from glycoproteins on virions, which allows virus release and prevents virion aggregation on the host cell where they are produced (8).

4.2 SARS-CoV-2 Release

The exact mechanism of SARS-CoV-2 release is still being defined. However, it is proposed that it uses the endoplasmic reticulum-trans Golgi intermediate compartment (ERGIC) to form mature virions. Those virions then translocate to the cell surface in vesicles formed from budding through the ERGIC membrane, followed by release via exocytosis. Interestingly, a furin cleavage site was discovered in the SARS-CoV-2 spike protein, which is absent in SARS-CoV, thatmight play an important role in viral egress (33). The cleavage recognition motif is similar to that of MERS-CoV, which is likely cleaved by furin during virus egress (34).

4.3 Antivirals

To date the only recommended influenza antivirals by the WHO are NA inhibitors, which prevent the release of the viral progeny from the infected host cell. NA) removes sialic acids from host cells and from glycoproteins on virions, which allows virus release and prevents virion aggregation respectively(8). The inhibitors bind to the active site of the viral NA proteins, being competitive analogs of sialic acid. Thus, NA inhibitors prevent the sialidase activity of NA. Currently three NA inhibitors are licensed globally for influenza A and B infections, namely oseltamivir, zanamivir and peramivir. Furthermore, laninamivir is licenced in particular countries, including Japan, where it is approved for the prevention and treatment of influenza A and B. Laninamivir is a single-dose long lasting NA inhibitor. Oseltamivir is administered orally, zanamivir is inhaled and peramivir is delivered intravenously. These antiviral drugs differ in chemical structure, side-effects, risks, metabolization and excretion.
A recurring problem for influenza antivirals is the emergence and dissemination of resistant mutants. As of now, all widely used and approved influenza antivirals focused on interference at the release step of the virus are NA inhibitors (35).
Currently, oseltamivir is in clinical trials phase 4 for Sars-CoV-2. This IAV antiviral has been used for treating COVID-19 patients in Chinese hospital, however its efficacy has not been proven yet. Moreover, the latest research points out that NA inhibitors, such as the ones mentioned before, have insufficient activity against SARS-CoV-2 (36). SARS-CoV-2 depends on sialic-acid containing glycoproteins acting as attachment factors, but the use of NA activity has not been

proven (37).
As seen in recent times, when a pandemic occurs, the first looked at treatment are antiviral agents. Mostly, antiviral agents that have been developed, tested and used for other viruses and diseases. Those drugs can be used immediately and thus are fastest to deploy. Yet, broadly neutralizing antibodies (bNAbs) have the potential of being a short-term prophylaxis or treatment. Numerous antibodies have shown in vivo efficacy against different Influenza A subtypes, such as H1N1, H3N2 and H2 viruses (38).
Chen et al. (2019) found that antibodies (MAb 045-2B06 and CR9114) that bind to the stalk of HA interfere with the release of influenza particles. The HA stalk-binding antibodies inhibit the NA activity of the virus particles. As mentioned before, NA inhibition (NI) prohibits release of the viral progeny from the infected cell. The HA stalk-binding antibodies inhibit NA enzymatic activity through steric hindrance. Remarkably, the paper also found that the same antibodies were less effective in neutralizing viral infection when they lacked the NA inhibition activity (39). Brandenburg et al. (2013) showed that also HA head-binding antibodies prohibited viral egress, while the stalk-binding antibodies used (CR6261 and CR8020) did not inhibit egress (40). Both papers tested the viruses on H1N1 and H3N2 subtypes.
Additionally, both antibodies CR9114 and CR6261 are effective in protecting mice from H1N1, H5N1, H3N2 and H2 subtypes (38), indicating that both HA stalk-binding antibodies and HA head-binding antibodies can inhibit viral egress, possibly through different mechanisms. Also, not all HA stalk-binding or HA head-binding antibodies have the same mechanism of interference Most antibodies for IAV target the head of HA. This head is highly variable, which makes the antibodies very strain specific, providing room for immune evasion by IAV. Even though there are studies on heterosubtypic neutralizing antibodies that target the receptor-binding site and thus the HA head, influenza has adapted well to protect the receptor-binding site. Subtypes have differences in loops and helices surrounding the receptor-binding site. This makes HA head binding antibodies generally more constrained in specificity than antibodies targeting the highly conserved HA stem region. These HA stem-binding antibodies prevent fusion of the host and virus membranes by preventing conformational changes associated with the fusion, thus inhibiting viral entry (41).
Discoveries on IAV showed that targeting conserved regions results in a broader specificity of antibodies. Combining this with current knowledge of SARS-CoV-2, furin could be a possible target of antiviral antibodies. Furin can be examined for conserved regions and the active binding site. Subsequent antivirals or antibodies against this protein could potentially be used, especially targeted towards conserved regions, in the treatment of SARS-CoV-2.
A study by Sutton et al. showed that early studies with two HA-stem binding antibodies (CR6261 and CR9114) exhibited weak in vitro activity against H2 influenza viruses. However, the in vivo efficacy was not tested. The antibodies were tested in vitro against human- and animal-origin H2 viruses and CR6261 neutralized both viruses and CR9114 only neutralized one. Then, prophylactic efficacy was examined in mice and both antibodies reduced mortality. This study clearly shows the need for in vivo evaluation of antibodies. CR9114 did not show neutralizing activity in vitro, however in vivo it did clearly show prophylactic efficacy. So, in the quest of finding neutralizing and prophylactic antibodies against SARS-CoV-2 it is very important to do in vivo evaluation (38).

5 Metabolic Pathways

As a consequence of advancements in proteomics, metagenomics and mostly metabolomics, studying the host cell metabolism upon viral infection presents a whole new area for discovery of novel antiviral targets. These virus induced metabolic changes are highly virus specific (42). Also, for virus induced metabolic changes there seems to be less relevance of comparability in viral pathogenesis and more correlation between virus genome type and implications on host cell metabolism (43). Therefore, in the case of relevant virus induced metabolism studies it appears more useful to regard viruses with highly similar genome types. Since IAV and SARS-CoV-2 differ in genome type, there are no studies specifically comparing metabolic changes induced by SARS-CoV-2 with those of IAV. It has been established that many viruses, including IAV (44, 45) and SARS-CoV-2 (46), have a significant impact on the host cell's metabolism. The most distinctive and researched metabolic alterations are: upregulation of fatty acid synthesis, inducing glycolysis, glutaminolysis and upregulation of the kynurenine pathway (42, 45, 47-49).

5.1 Metabolic Pathways Targeted by Viruses

5.1.1 Glycolysis

Glycolysis is a metabolic pathway where glucose is converted into pyruvate, then lactate – which is pumped out of the cell. The energy released is transferred to the two main energy carriers of cells; NADH and ATP. This is done by reducing NAD+ to NADH and phosphorylation of AMP resulting in ATP production. Under standard growth conditions mammalian cells utilize the pyruvate to drive the TCA cycle in the mitochondria which results in oxygen facilitated usage of the electron transport chain. This process, starting with glucose intake, is called oxidative phosphorylation. Under normal anaerobic conditions the pyruvate is, like with glycolysis, converted into lactate and pumped out of the cell. In cancer cells this is done regardless of the amount of oxygen present – also known as the Warburg effect (50). The former

process of oxidative phosphorylation provides significantly more ATP per glucose used. Glycolysis however provides ATP much more rapidly.

5.1.2 Upregulated Fatty Acid Synthesis

Upregulated fatty acid synthesis causes an increase in the creation of lipids, which are a critical component for increased membrane production. In mammalian cells, citrate, an intermediate from the TCA cycle, is mostly used to synthesize the carbon substrates needed for fatty acid synthesis. Production of Palmitate from Acetyl-CoA and Malonyl-CoA is the main reaction in fatty acid synthesis. The reaction is catalyzed by fatty acid synthase (FASN) and uses NADPH. The end products are long chain fatty acids that can be used for lipid production, used in membranes or lipid droplet formation. These long chain fatty acids are also essential for viable virion production (51) and can be a source of energy by breakdown through beta-oxidation. An example of upregulated fatty acid synthesis is observed through increased FASN expression, the previously mentioned catalyst of de novo synthesis of fatty acids, in Hepatitis C virus (HCV) infected human hepatoma cell line (47).

5.1.3 Glutaminolysis

Glutaminolysis is the process of converting glutamine into glutamate and then α-ketoglutarate. Glutamine is an important type of metabolic fuel for rapidly growing/producing cells, such as virion producing infected cells. It is used for many cellular metabolic pathways including ammonia and glutathione production. A-ketoglutarate is a critical metabolite that is used in both replenishing TCA cycle intermediates and ATP production. It does this through diversion of glucose carbon sources away from the TCA cycle into lactic acid production and fatty acid synthesis, a result of the previously mentioned glycolysis and upregulated fatty acid synthesis respectively. Increased glutamine uptake through inducing glutaminolysis is therefore a key virus induced metabolic alteration that provides the energy and carbon biomass needed for virion production (52-54).

5.1.4 Upregulated Kynurenine Pathway

Kynurenine is a metabolite formed in tryptophan catabolism by the enzyme indoleamine 2,3-dioxygenase (IDO). This enzyme is normally only expressed in low levels but significantly upregulated in cancer cells and virus infected cells (55, 56). Increased IDO expression and subsequent upregulation of the kynurenine pathway has a wide range of effects ranging from inducing psychiatric diseases to controlling blood pressure and, most relevantly, attenuating the immune response (57-59).

5.2 Advantages of Virus Induced Host Cell Metabolic Alterations

These viruses induced metabolic alterations seem to serve the increased need for rapid energy turnover and altered carbon sources needed for virion production and assembly, including amino acids, free nucleotides and long chain fatty acids. Inhibition of the synthesis of either of these carbon sources or rapid energy turnover pathways leads to decreased virion production (60). The upregulation of the kynurenine pathway specifically suppresses the immune response by inhibiting T-cell response (59). Together these findings suggest that these metabolic alterations can be potential antiviral targets.

5.3 Antivirals

5.3.1 Effective Metabolic Antiviral Targets

When targeting glycolysis or glutaminolysis one is inhibiting a part of the TCA cycle. However, this is not advisable since using general cellular process inhibitors can lead to critical metabolic complications in oxidation-dependent cells, such as neural stem/progenitor cells (NSPCs) (61). For glycolysis inhibitors the apparent toxicity that most glycolysis inhibitors, like pentavalent arsenic, represent has been circumvented. This was done by usage of the nontoxic synthetic glucose variant 2-deoxy-d-glucose (2-DG) which inhibits hexokinase II (HKII); a catalyst of the initial metabolic step in glycolysis (62). With targeting of the fatty acid synthesis there are less serious complications. This is because even though fatty acids are important in development and growth, they are not immediately essential in mammals (63-65). Meaning impairment of fatty acid synthesis, during treatment for example, will not be immediately fatal and is therefore a viable strategy. Targeting the kynurenine pathway could be useful as a supplementary antiviral, since it can potentially restore host organism immune responses. Unless highly spatially coordinated drug delivery or activation is involved, targeting glycolysis or glutaminolysis is currently non-viable, with the exception of targeting using 2-DG. Therefore glycolysis - through inhibition using 2-DG, upregulation of fatty acid synthesis and the kynurenine pathway currently present the most viable metabolic antiviral targets.

5.3.2 Existing Metabolic Antivirals

As mentioned before, upregulation of fatty acid synthesis currently represents one of the most viable metabolisms related antiviral targets. Therefore, most promising metabolic antivirals are some form of fatty acid synthesis inhibitors. Examples of existing treatments targeting virus induced upregulation of fatty acid synthesis are; “rotavirus (RV) infected cells treated with C75, an inhibitor of the fatty acid

synthase enzyme complex (FASN), reduced RV infectivity 3.2-fold (P = 0.07)", "TOFA [5-(Tetradecyloxy)-2-furoic acid] inhibits the enzyme acetyl-CoA carboxylase 1 (ACC1). TOFA reduced the infectivity of progeny RV 31-fold" (60), inhibitors of NS5A, such as daclatasvir, blocking replication of Hepatitis virus C (HCV) RNA at the stage of membranous web biogenesis (66) and Fluvastatin inhibiting HCV replication through microtubule bundling and a doublecortin-like kinase-mediated mechanism (67).
Other existing metabolic antivirals target glycolysis through treatment with 2-DG which abolished RV replication in primary human fibroblasts and in HeLa cells (68). Furthermore, targeting the kynurenine pathway through IDO inhibitors has proven effective. This has antiviral potential by targeting IDO-induced tryptophan depletion, which prevents suppression of the T-cell immune response (45, 59).

5.3.3 Relevant Metabolic Antivirals SARS-CoV-2

There is currently no decisive answer on what dictates effectiveness and broadness of application for treatments using metabolism related antivirals. This is in part because the exact mechanisms of viral induced metabolic alterations are still largely unknown. Moreover, there are hardly any universal virus induced host cell metabolic alterations, since the metabolic alterations are both highly virus type and host cell type specific (42). Therefore it seems likely that metabolic antivirals relevant to SARS-CoV-2 should be developed for highly similar virus and host cell types (69). Fortunately, there is a prevalent group of viruses with a highly similar genetic structure that have metabolic antivirals that are being developed or have been developed, namely viruses of the class Filoviridae. Filoviridae are, like SARS-CoV-2, enveloped single stranded positive sense RNA based viruses, which could therefore be a useful model for treatment of SARS-CoV-2 infection. This concept that similarity in virus type leads to similar virus induced host cell metabolic alterations has actually been reviewed in the case of membranous web biogenesis. It was shown that HCV-infected cells have similar membranous web biogenesis and structure to those of the similar in virus type, but unrelated picorna-, corona- and arteriviruses, although distinct from closely related flaviviruses (43). This shows that correlation of virus type to host cell metabolism might be present and that broader application of specific antivirals for similar virus types could be effective. An example of an existing antiviral metabolism treatment of similar virus type being applied to SARS-CoV-2 is 2-DG. 2-DG prevents SARS-CoV-2 replication in Caco-2 cells by blocking glycolysis (46).
Examples of other treatments that could be applicable to SARS-CoV-2 are the previously mentioned inhibitors of NS5A, such as Daclatasvir, acting on membranous web biogenesis (66) and Fluvastatin,impairing replication through microtubule bundling and a doublecortin-like kinase-mediated mechanism - which regulates microtubule polymerization (67).

6 Discussion

The results of the research as laid out in the previous section show an extensive description of the viral life cycle parts. In addition, the research results allow us to formulate an overview for SARS-CoV-2 of the applicability of various existing antiviral approaches interfering at several parts in the viral life cycle. The therapeutic strategies and research methods have been outlined and explained in depth.
Regarding to the entry part of the viral life cycle, the membrane proteins for Influenza A and SARS-CoV-2 differ and therefore the entry part contains another receptor. Zanamir and oseltamivir were earlier discussed antivirals that block the entry for Influenza A and also inhibit this particular step in MERS-CoV. As MERS-CoV is similar to SARS-CoV-2 in its structure, referring to the membrane proteins, it can be assumed that these antivirals can potentially work for inhibition SARS-CoV-2 entry into host cells. Additionally, the drug arbidol is an Influenza antiviral that has already been proven as COVID-19 treatment. Despite the differences in the structure and entry part of Influenza A and SARS-CoV-2, it seems that drugs of influenza in this part are applicable to SARS-CoV-2.
The research of viral replication part in Influenza A and SARS-CoV-2 have elucidated several differences among these viruses. Namely, Influenza A is a negative-sense RNA virus and the replication takes place in the nucleus, whereas SARS-CoV-2 is a positive-sense RNA virus, the replication takes place in the cytosol and contains a cap snatching process. Several antivirals were outlined that target this specific cap snatching process in Influenza A in the replication step. It can be concluded that these antivirals are not applicable to SARS-CoV-2 as this interference is irrelevant in the replication step of the SARS-CoV-2 viral life cycle. Nevertheless, there seems to be potential in the applicability to SARS-CoV-2. The drug favipiravir is a broad-specificity drug for multiple RNA viruses, such as non-seasonal influenza viruses, that specifically interferes at the synthesis of the viral RNA. It acts as purine analogue and causes mutations without affecting host cellular DNA or RNA. The application of favipiravir on infected SARS-CoV-2 cells has demonstrated that there was an increase in mutation frequency. The drug favipiravir has also been tested clinically on SARS-CoV-2 patients and the outcome of these tests were very promising as they showed an increase in the recovery rates of the patients. Additionally, the symptoms of the SARS-CoV-2 patients were also reduced. Although further testing is needed, favipiravir is a very promising antiviral for blocking the viral RNA synthesis that can be applicable to SARS-CoV-2. Another promising potential antiviral ap-

proach is based on the use of the CRISPR mechanism, where Abott et al. (2020) designed CRISPR RNAs (crRNAs) that specifically target the viral RNA and degrade it. Based on the experiments it was proven and found that the designed crRNAs with the corresponding CRISPR strategy were capable of targeting 92% of 91.600 IAV strains and 91% of 3051 coronaviruses (70). Despite the fact that this has a very high potential for an effective antiviral approach, this strategy is still in early stages and needs a lot of further testing to prove its applicability in the clinic.

As stated already, the exact mechanism and understanding of the SARS-CoV-2 release is still not complete. Nevertheless, it seems that the release step of the viral life cycle is similar only for the ''budding" part. For Influenza A this occurs when the virus particles are about to leave the host cell, and therefore the virus particles execute this step at the host cell membrane. The viral envelope is acquired from the host cell membrane and then simultaneously the virus particles leave the host cell, whereas the budding for SARS-CoV-2 virus particles occurs earlier through the ERGIC membrane. Thereafter, SARS-CoV-2 virus particles leave the host cell via exocytosis. Earlier it was stated that the viral protein NA of influenza A is important for the release step in the viral life cycle. Interference in this particular step is regulated by, WHO-approved, NA inhibitors that disrupt the viral release. This step is not applicable to SARS-CoV-2 as there is a low efficacy of interaction with the NA inhibitors and the virus does not contain NA proteins. As indicated previously, the underlying mechanism of the viral release for SARS-CoV-2 is not completely outlined yet and therefore this step should be further analysed in the future. Contrarily, the Influenza A virus antibodies show that targeting the conservative regions of membrane proteins makes the antibodies less strain and subtype specific, which can help prevent the outbreak of a new epidemic of the same virus from a different subtype. Therefore, for SARS-CoV-2 it is very useful to produce antibodies that target conservative regions of membrane proteins or other targets that are not highly variable among subtypes. Although completely universal virus induced metabolic changes are currently not clearly defined, there are well defined virus specific metabolic changes. Further assaying of these changes do have potential for valuable new insights on the effect of viral infection and possible antiviral treatments. Furthermore, future testing of metabolism related antiviral treatments on SARS-CoV-2 infected cells might offer insights on how broadly these virus specific antivirals can be applied. Additionally, this could prove to be a powerful tool in attenuating viral replication. Also, when used in tandem with other antivirals it can help in designing effective antiviral therapies.

This research elucidated that several antivirals already developed for Influenza A interfering in the viral life cycle can be applicable to SARS-CoV-2. It was shown that looking into the viral life cycle steps and developing antivirals of Influenza A - that has caused pandemics before - can be an example for forming an effective strategy in order to respond quickly to future pandemics. Additionally, the outcomes of this research provide guidelines to develop a roadmap of interfering with the viral life cycle steps to contain future outbreaks, especially of other coronaviruses.

The focus of further research should be on what the underlying scientific reason is for the success of previous treatments. Seeking to understand what the exact mechanism of action is of the interference with the specific viral life cycle step of developed antivirals. A good example of such a research is by Chen et al. (2019) who found HA-stalk binding antibodies utilize NA inhibition through steric hindrance. By clarifying these exact mechanisms, the applicability to other viruses can be more well defined based on their molecular structure and the processes they use. This could help with faster and better selection of possible antivirals when a new pandemic occurs, focusing the research on the more relevant antivirals. Although unraveling these mechanisms is far from easy, it is worth investing time in such studies. This greater understanding and deep insight are needed to develop such a roadmap.

7 Author Contributions

H. Hulsewé constructed the introduction. P. Kostina drafted the entry part for SARS-CoV-2 and influenza A. A. Huijsmans drafted the replication of the viral life cycle part in this report for SARS-CoV-2 as well as for influenza A. Besides, A. Huijsmans was responsible for the coherence of all the created parts in this report. L. Hofmans was responsible for the release in the viral cycle part of this report for SARS-CoV-2 and influenza A. F. Jansen drafted the abstract and the metabolic pathways part of this report and made the table that corresponds to the graphical abstract. R. Niemeijer contributed to the abstract part and created the graphical abstract where the findings of the other authors were put in. Additionally, R. Niemeijer drafted the discussion of the report. A. Huijsmans and R. Niemeijer were in charge of this project and acted actively in planning the meetings and dividing the tasks and responsibilities among the authors. All authors determined the structure of the report together.

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APPENDIX A

Viral life cycle process	Antiviral target	Mode of inhibition	Existing treatment	New treatment in development	Applicable to SARS-CoV-2	Source paper
Entry	HA	Blocking viral fusion with host cell membrane	Umifenovir	-	Likely	(Wang et al., 2020)
Replication	RdRp	Purine analogue incorporated into viral RNA during genome replication	Favipiravir	-	Yes	(Mifsud et al., 2019)
Release	Neuraminidase protein	Competitive analog of neuraminidase target: sialic acid	Oseltamivir, Zanamivir and Peramivir	-	Unlikely	(Yousefi et al., 2020)
Release	HA-stalk	Steric hindrance of NA enzymatic activity	-	-	Unlikely	(Chen, yu-ling Lan & Huang, 2019)
Host cell metabolism	Fatty acid synthesis	Membranous web biogenesis	Daclatasvir	More inhibitors of NS5A	Likely	(Berger et al., 2014)
Host cell metabolism	Fatty acid synthesis	Microtubule bundling and a doublecortin-like kinase-mediated mechanism	Fluvastatin	Other statins	Likely	(Ali et al., 2013)
Host cell metabolism	Glycolysis	Blocking glucose intake	2-DG	2-DG applied to SARS-CoV-2	Yes	(Gualdoni et al., 2018), (Bojkova et al., 2020)

Table 1: Overview of found antiviral targets within the viral life cycle. Naming their: mode of inhibition, existing treatement(s), possible new treatement(s) in development, applicability estimate for SARS-CoV-2 and the source paper.