Biomimetic Strain-Stiffening Self-Assembled Hydrogels

Wang, Yiming; Xu, Zhi; Lovrak, Matija; le Sage, Vincent A.A.; Zhang, Kai; Guo, Xuhong; Eelkema, Rienk; Mendes, Eduardo; van Esch, Jan H.

DOI
10.1002/anie.201911364

Publication date
2020

Document Version
Accepted author manuscript

Published in
Angewandte Chemie - International Edition

Biomimetic strain-stiffening self-assembled hydrogels

Yiming Wang[1], Zhi Xu[1], Matija Lovrak[2], Vincent A. A. le Sage[2], Kai Zhang[2], Xuhong Guo[1], Rienk Eelkema[2], Eduardo Mendes[2], Jan H. van Esch[2]*

State Key Laboratory of Chemical Engineering, School of Chemical Engineering, East China University of Science and Technology, Shanghai 200237, China

Department of Chemical Engineering, Delft University of Technology, van der Maasweg 9, 2629 HZ, Delft, The Netherlands

Correspondence email: j.h.vanesch@tudelft.nl

Abstract: Supramolecular structures with strain-stiffening property are ubiquitous in nature, but remain rare in the lab. Here we report on strain-stiffening supramolecular hydrogels that are entirely produced through self-assembly of synthetic molecular gelators. The involved gelators self-assemble into semi-flexible fibers which thereby crosslink into hydrogels. Interestingly, these hydrogels are capable of stiffening in response to applied stress, resembling biological intermediate filaments system. Furthermore, strain-stiffening hydrogel networks embedded with liposomes are constructed through orthogonal self-assembly of gelators and phospholipids, closely mimicking biological tissues in both architecture and mechanical properties. This work furthers the development of biomimetic soft materials with mechanical responsiveness and presents potentially enticing applications in broad fields, such as tissue engineering, artificial life, and strain sensors.

Biological systems have the capacity to actively respond to external mechanical forces, with the aim, among others, to protect themselves from the damage led by large deformations.[1] Such a mechanical responsiveness is referred to as strain-stiffening which is defined as an increase in the stiffness of a system with applied strain. The strain-stiffening behavior of biological systems is provided largely by their cytoskeleton structures. These cytoskeleton consist of crosslinked semi-flexible biopolymers,[2] such as collagen fibers,[3] actin filaments (F-actin),[4] and fibrins.[5] Interestingly, in nature, these biopolymers are entirely formed by self-assembly of the corresponding protein subunits. Inspired by biomolecular systems, in recent decades, a mass of synthetic supramolecular gels bearing fibrous matrix structures have been prepared via self-assembly of small molecular building blocks[6] with a few of them even well-suited for clinical applications.[7] However, when mimicking living tissue, these synthetic supramolecular gels lack of exhibiting strain-stiffening behavior, a fundamental property of living tissue.

To mimic the strain-stiffening behavior, a series of theoretical models have been established to understand the stiffening mechanism.[8] These theoretical studies suggest that strain-stiffening, in principle, can be observed in all semi-flexible polymers crosslinked materials for which the bending modules of the assemblies is comparable to the thermal energy, k_BT. The lack of observation in synthetic systems can be ascribed to the challenge to prepare self-assembled structures composed of semi-flexible fibers that are thin enough and sufficiently crosslinked that allow the matrix structures to access their stiffening regimes before rupture.[9] So far, very few examples of synthetic semi-flexible filaments leading to strain-stiffening materials have been reported. Kouwer and his colleagues presented the first example of synthetic strain-stiffening hydrogels that are formed by the entanglement of semi-flexible polyisocyanopeptides (PICs) bundles, closely mimicking the characteristic mechanical properties of biological intermediate filaments.[10] The other example was described very recently by Sijbesma.[9] In their work, strain-stiffening hydrogels are prepared by covalently crosslinking of semi-flexible rod-like micelles that are formed through self-assembly and covalent fixation of oligo(ethylene oxide) grafted bisurea bolaamphiphiles. In the present work, we describe a new class of strain-stiffening supramolecular hydrogel materials that are formed entirely through self-assembly of synthetic molecules.

In recent years, we have developed a hydrazone-based modular gelator system in which neutral gelators (**NGs**: **HA$_3$**) are formed in situ from the building blocks of hydrazide (**H**) and neutral aldehyde **A** through formation of hydrazone bonds (Figure 1a).[11] The generated **NGs** self-assemble into semi-flexible neutral fibers (**NFs**) which subsequently crosslink into hydrogel networks through physical interfibrous interactions and entanglements (Figure S1). However, the resulting hydrogels do not show stiffening with applied strain (Figure S2), which can be explained by the formation of bundles (Figure S1). While bundling would significantly enhance the stiffness of the fibers,[8c] as a result, the

physical crosslinks are not sufficiently strong to permit these stiff bundles to enter their athermal stiffening regimes.[9]

To address this problem and avoid bundling, we propose to decorate the fibers with electrically charged groups with the aim to prevent the occurrence of bundling relying on interfibrous electrostatic repulsion. For this purpose, besides the addition of **A**, an anionic aldehyde (**A**$^{-}$) is added to the system to induce the formation of a mixture of gelators consisting of **NGs** and charged gelators (**CGs**: **HA$_2$A$^-$**, **HAA$^-_2$**, **HA$^-_3$**) (Figure 1a).[12] Charged fibers (**CFs**) are obtained through a seeding-driven co-assembly of **NGs** and **CGs** (Figure 1b).[13] We find that homogeneous hydrogels composed of **CFs** are formed. Importantly, these new homogeneous gels show interesting strain-stiffening behavior resembling the mechanical properties of other biological strain-stiffening networks. Furthermore, more complex supramolecular structures consisting of coexisted strain-stiffening fibrous networks and liposomes are created through orthogonal self-assembly of these gelators and phospholipids, closely mimicking biological tissues in both the architectures and the mechanical properties (Figure 1c).

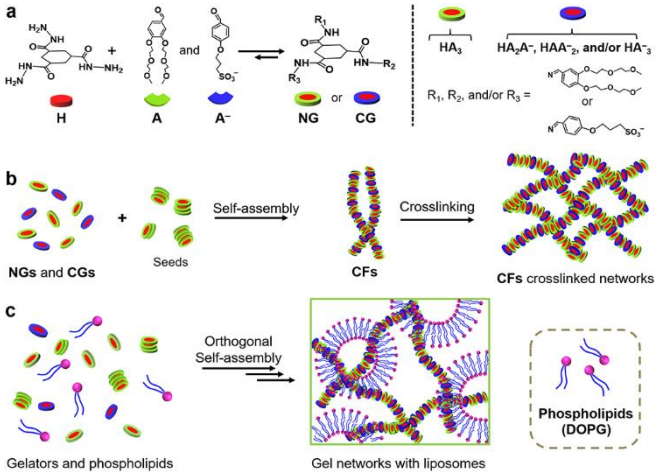

Figure 1. a) Scheme of formation of gelators (**NGs** and **CGs**) from the molecular building blocks (**H**, **A**, and **A**$^{-}$); b) seeding-driven co-assembly of **NGs** and **CGs** leading to **CFs** crosslinked hydrogel networks; and c) orthogonal self-assembly of the gelators and phospholipids towards complex supramolecular structures consisting of coexisted hydrogel networks and liposomes.

All the gel samples were prepared from phosphate buffered stock solutions (0.1 M, pH 7.0) of **H**, **A**, and **A**$^{-}$. The total concentration of aldehydes (**A** + **A**$^{-}$) was constantly kept at six times higher than **H** to achieve a complete conversion of **H** into tris-hydrazone gelators. To initiate the co-assembly of **NGs** and **CGs**, seeds composed of 1.0 mM **NGs** were added (see supporting information).[13] An aldehyde functionalized fluorescein derivative (**A-FL**) (green, Figure S3) was used to label all the self-assembled structures; and a cationic dye, **Hoechst 33342** (blue, Figure S3), was used to label the **CFs**. The fluorescent probe labelled structures were characterized by confocal laser scanning microscopy

(CLSM). The phospholipids used in this study was **DOPG** (1,2-Dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) and the self-assembled liposomes were labeled using a lipid decorated with rhodamine (**L-Rh**, red) (Figure S3).

We found that the presence of $\mathbf{A}^-$ led to an increase in the transparency of the resulting gel samples (Figure S4), and the gel became completely transparent with addition of 30 mol% $\mathbf{A}^-$ (Figure 2a and S4). CLSM observations (green channel) showed that the dimensions of the crosslinked gel fibers were effectively reduced with the content of $\mathbf{A}^-$, varying from large clusters with 0 mol% $\mathbf{A}^-$ to thin bundles with 20 mol% $\mathbf{A}^-$ (Figure S5), and further to completely homogeneous gel networks with 30 mol% $\mathbf{A}^-$ of which the microscopic structures cannot be resolved by CLSM (Figure 2a). Cryo-TEM studies revealed that such a homogeneous gel network was composed of nano-sized thin fibers with a diameter of ~5.8 nm (Figure 2b) which is comparable to the single hydrazone fibers.[11, 13] In the blue channel of CLSM, we found that the intensity of blue fluorescence increased with the content of $\mathbf{A}^-$, indicating the increase in the content of charges of the fibers (Figure S5). For the gels prepared with 30 mol% $\mathbf{A}^-$, a completely homogeneous blue fluorescence was observed (Figure S5), which therefore demonstrates that the thin fibers are **CFs**. Indeed, the electrostatic interactions between **CFs** would effectively prevent the occurrence of bundling, thereby resulting in the homogeneous gel networks.[13]

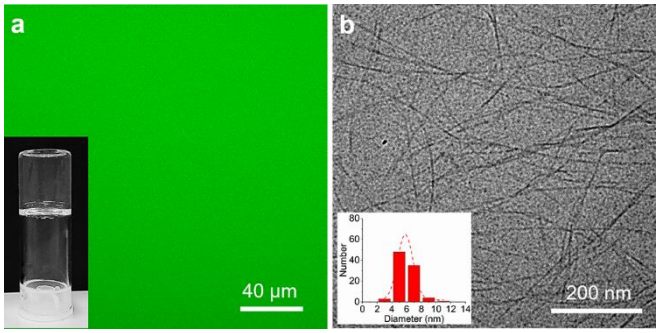

Figure 2. a) Confocal images of the gel networks, inset in a) is photograph of the gel sample; and b) cryo-TEM image of the gel fibers, inset graph is the diameter distribution of the fibers. Samples are prepared from [**H**] = 20 mM, [**A** + $\mathbf{A}^-$] = 120 mM (30 mol% $\mathbf{A}^-$), [**A-FL**] = 30 μM, [**Hoechst 33342**] = 20 μM, and seeds consisted of 1.0 mM **NGs**.

Next, we employed oscillatory rheology to investigate the mechanical properties of the gels prepared with addition of $\mathbf{A}^-$ (see supporting information). The results showed that the gelation time, defined as the time at which the storage modulus G' surpasses the loss modulus G", was increased from ~8 to ~42 min with increasing the content of $\mathbf{A}^-$ from 0 to 30 mol% (Figure S6). Correspondingly, the stiffness of the resulting gels was reduced from ~10.8 kPa to ~ 2.7 kPa (Figure S6). The longer gelation time and softer gels with higher $\mathbf{A}^-$ content can be explained by the increased electrostatic repulsions between the gelators or fibers which therefore limit the self-assembly of gelators or crosslinking of fibers, without changing significantly the persistence length of single fibers via addition

of charged groups. Frequency sweep measurements demonstrated that *G′* of the gels was constantly higher than *G″*, and both *G′* and *G″* were independent of frequency (Figure S7), indicating the stable solid feature of these hydrogels.

To examine the mechanical behavior of the gels under applied strain, we further performed strain sweep tests on the formed gels. The results showed that the gels prepared with < 20 mol% $\mathbf{A}^-$ were destroyed directly after a linear regime upon the increase in strain. Interestingly, we found that the critical strain, at which the gels undergoes a transition from linear regime to non-linear regime, shifted from ~0.1% with 0 mol% $\mathbf{A}^-$ to ~1.2% with 20 mol% $\mathbf{A}^-$ (Figure S8). We attribute this critical strain shift to the decrease in the sizes of the bundles with increase in $\mathbf{A}^-$ content as we observed in CLSM (Figure S5). The resulting thinner bundles are relatively softer than the thicker bundles, which are capable of dissipating more stress through bending and therefore enable the gel networks to tolerate a larger deformation before breaking. To our surprise, upon a further increase in the content of $\mathbf{A}^-$ (≥ 20 mol%), the resulting gels presented strain-stiffening behavior after the linear regime instead of the aforementioned softening (Figure S8). This can be explained by the further decrease in the sizes of the bundles (Figure S5) by which the stiffness of the semi-flexible fibers (bundles) is reduced to a level that is comparable to the thermal agitation energy, thereby activating the stiffening regime of the fibers. This also implies that the stiffening of our gels is led by exhaustion of soft bending modes.[3c] Moreover, the critical strain, at which the gels start to enter stiffening state, increased from ~1.2% (20 mol% $\mathbf{A}^-$) to ~1.3% (25 mol%), and almost leveled off at ~1.5% when the content of $\mathbf{A}^-$ reaches 30 mol% (Figure S8). This means that the sizes of the bundles were further decreased with increasing the $\mathbf{A}^-$ content and reached the lowest level (single hydrazone fibers) with addition of ~30 mol% $\mathbf{A}^-$. Indeed, preceding cryo-TEM experiments have confirmed that the gel prepared with 30 mol% $\mathbf{A}^-$ is mostly composed of single hydrazone fibers (Figure 2b). The most striking stiffening behavior was observed in the gel prepared with 30 mol% $\mathbf{A}^-$. It is likely that in this case the gel networks are mostly composed of single hydrazone fibers, therefore each fiber is allowed to bend and contribute to the stiffening. However, the stiffening behavior became less prominent with a further increase in the content of $\mathbf{A}^-$ (35 mol%). This can be caused by the decreased crosslinking degree of the fibers resulting from larger electrostatic interfibrous repulsions. The weak crosslinks cannot permit a sufficient deformation of the fibers, thereby leading to weakening of the stiffening behavior. In the following strain-stiffening studies, we only focus on the gel samples prepared with 30 mol% $\mathbf{A}^-$, as the resulting gel networks are uniform and show obvious stiffening property.

To obtain further insight into the strain-stiffening hydrogels, the differential modulus $K' = \mathrm{d}\sigma/\mathrm{d}\gamma$ (where σ is the shear stress, and γ is the applied strain) of the gels prepared with different concentrations of **H** (*c*) (the relative contents of other species are kept constantly) was recorded on the basis of a previously reported method.[3c] We found that transparent gels are nicely formed at

varying *c* from 5 to 20 mM (Figure 3a). In the lower stress regime, *K'* is equal to the storage modulus in linear region G_0 (Figure 3b). G_0 was increased with *c* (Figure 3b), showing a correlation of $G_0 \sim c^{1.8}$ (Figure S9) which is comparable to the concentration dependence of collagen fibers.[3a, 3c, 4a, 14] Interestingly, in the high stress regime, all the *K'* curves collapsed to a single master curve, showing a stiffening exponent n = 1.0 (Figure 3b and 3c). Such a strain-stiffening property resembles the mechanical properties of neurofilaments and type I collagen fibers (Table S1, see supporting information). In biological system, stiffening with n = 1.0 has been argued to be caused by the bending of semi-flexible filaments, which further suggests that the stiffening of our gels originates from the soft bending of the hydrazone fibers. Additionally, the stiffening range was reduced with increasing *c* because higher *c* leads to denser gel networks and increased confinement of the fibers, thereby effectively limiting the softer response of the fibers. Moreover, the critical stress of the stiffening σ_c showed a linear dependence of *c*, i.e. $\sigma_c \sim c^{1.8}$, which enables the rescaling of the *K'* versus σ curves to G_0 and σ_c, respectively. We found that all the rescaled curves corresponding to different *c* overlapped at a single master curve, indicating that all gel samples undergo a common stiffening mechanism.[3c, 9-10]

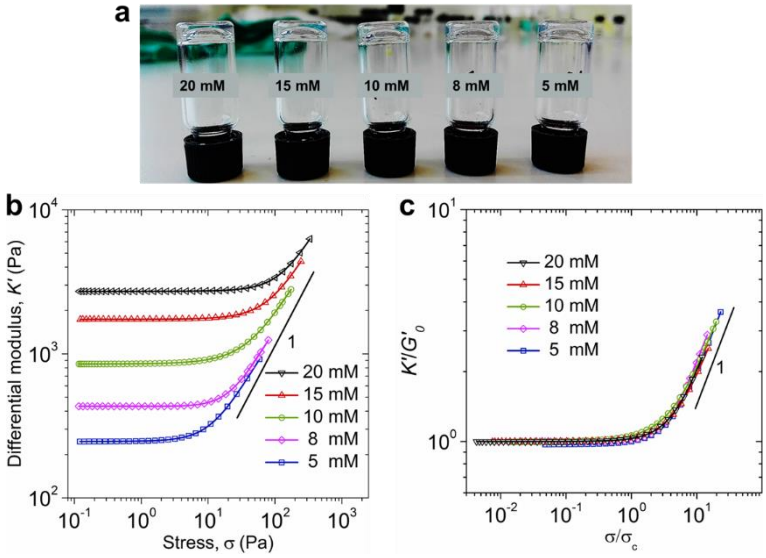

Figure 3. a) photographs of the gel samples at various concentrations *c*; b) differential modulus *K'* of the gels with varying concentrations as a function of applied stress σ; and c) rescaling of *K'*~σ curves with leveled off modulus G_0 and the critical stress σ_c, leading to a single master curve independent of gel concentrations.

Biological tissues are mainly composed of living cells embedded in the extracellular matrix (network), forming complex structures through the self-assembly of biological building blocks, including proteins and phospholipids. In recent years, various groups have tried to mimic the fundamental structures of biological tissues by performing so called orthogonal self-assembly of molecular building blocks, [15] producing for instance lipid vesicles embedded in a self-assembled gel

network.[16] However, none of them show strain-stiffening like their biological counterparts.

Here, with a strain-stiffening supramolecular hydrogel at hand, we are curious to see whether we can build a complex supramolecular system that mimics biological tissues in both the architectures and mechanical properties. To this end, we constructed hybrid materials by orthogonal self-assembly of the above studied gelators and **DOPG** phospholipids (see supporting information). We found that the resulting hybrid gel networks consist of coexisting homogeneous gel networks and giant liposomes with a diameter of ~16 μm (Figure S10, Figure 4a and Movie S1). The higher density of fibers in the areas of liposomes is caused by the local formation and self-assembly of hydrazone gelators due to the local catalysis of the phospholipid bilayers which has been investigated in a previous study.[17] After the construction of such tissue-like structures, we further investigated the mechanical properties of the resulting hybrid gels by recording the evolution of differential modulus *K'* against the applied stress *σ*. We found that these hybrid gels showed a similar strain-stiffening behavior as the preceding pure gels. All the curves in the stiffening regimes collapsed to a single master curve with a characteristic exponent of ~1.0. Furthermore, rescaling *K'* to G_0 and *σ* to σ_c led to a universal curve for all the *K'* ~ *σ* curves measured at different *c* (Figure 4c). These results confirm that the formation of embedded liposomes in the gel did not alter the mechanical responsiveness of the gel networks, and this artificial heterogeneous supramolecular system largely mimics biological tissues not only in the architectures, but also in the strain-stiffening behavior.

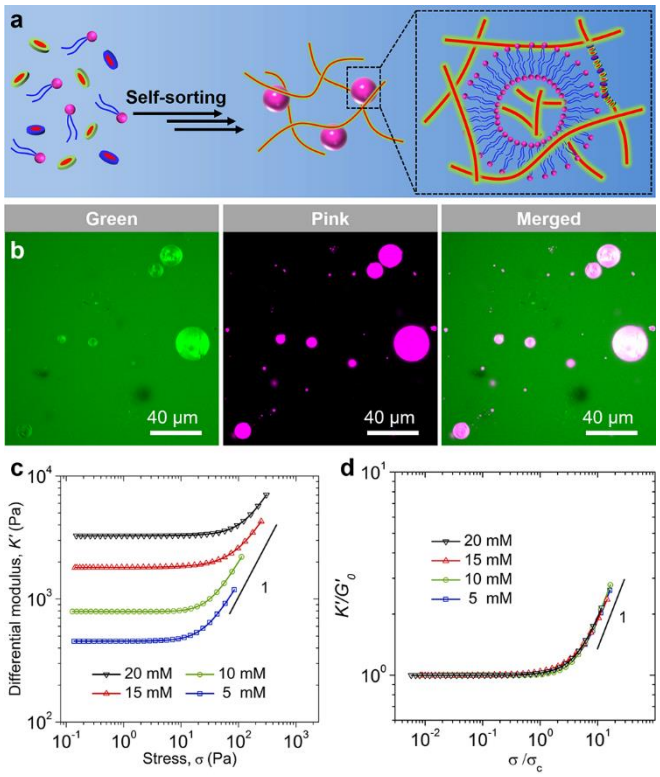

Figure 4. a) Confocal images in different channels showing the gel networks (green) and embedded giant liposomes (pink); b) differential modulus *K'* of the gels containing liposomes as a function of applied stress *σ*;

and c) rescaling of K' with the leveled off modulus G'_0 and σ with the critical stress σ_c, leading to a single master curve independent of gelator concentrations.

In conclusion, we have shown a type of biomimetic strain-stiffening supramolecular hydrogels that are formed entirely by the self-assembly of synthetic gelators. These hydrogels show comparable stiffening parameters and undergo a similar stiffening mechanism as compared to some biological intermediate filaments systems. Furthermore, strain-stiffening hydrogel networks embedded with micrometer-sized liposomes have been successfully created through the orthogonal self-assembly of the gelators and phospholipids. The resulting complex supramolecular structures open the door for a more realistic mimic of biological tissues in both the architectures and mechanical properties. Since the presented gelators can be easily decorated with functional groups due to their modularity,[18] we believe this mechanically responsive supramolecular system would have useful applications in various fields, for instance, tissue engineering. Furthermore, this complex strain-stiffening system can serve as a model system for the fundamental study on the deformation of vesicles or artificial cells to a local strain-stiffening environment.

Acknowledgment

We thank Dr. J. M. Poolman and Dr. C. Maity for providing **H**, **A**, $\mathbf{A}^-$, and **A-FL**. We would like to acknowledge China Scholarship Council (CSC) for financial support.

References

[1] a) J. D. Humphrey, *P Roy Soc a-Math Phy* **2003**, *459*, 3-46; b) A. R. Bausch, K. Kroy, *Nat. Phys.* **2006**, *2*, 231-238; c) K. E. Kasza, A. C. Rowat, J. Y. Liu, T. E. Angelini, C. P. Brangwynne, G. H. Koenderink, D. A. Weitz, *Current Opinion in Cell Biology* **2007**, *19*, 101-107.

[2] C. Storm, J. J. Pastore, F. C. MacKintosh, T. C. Lubensky, P. A. Janmey, *Nature* **2005**, *435*, 191-194.

[3] a) I. K. Piechocka, A. S. G. van Oosten, R. G. M. Breuls, G. H. Koenderink, *Biomacromolecules* **2011**, *12*, 2797-2805; b) S. Motte, L. J. Kaufman, *Biopolymers* **2013**, *99*, 35-46; c) A. J. Licup, S. Munster, A. Sharma, M. Sheinman, L. M. Jawerth, B. Fabry, D. A. Weitz, F. C. MacKintosh, *Proc Natl Acad Sci U S A* **2015**, *112*, 9573-9578; d) S. van Helvert, P. Friedl, *Acs Applied Materials & Interfaces* **2016**, *8*, 21946-21955.

[4] a) M. L. Gardel, J. H. Shin, F. C. MacKintosh, L. Mahadevan, P. Matsudaira, D. A. Weitz, *Science* **2004**, *304*, 1301-1305; b) M. L. Gardel, F. Nakamura, J. H. Hartwig, J. C. Crocker, T. P. Stossel, D. A. Weitz, *P Natl Acad Sci USA* **2006**, *103*, 1762-1767; c) E. M. Huisman, T. van Dillen, P. R. Onck, E. Van der Giessen, *Phys Rev Lett* **2007**, *99*.

[5] a) Q. Wen, A. Basu, J. P. Winer, A. Yodh, P. A. Janmey, *New J Phys* **2007**, *9*; b) H. Kang, Q. Wen, P. A. Janmey, J. X. Tang, E. Conti, F. C. MacKintosh, *J. Phys. Chem. B* **2009**, *113*, 3799-3805; c) M. R. Falvo, O. V. Gorkun, S. T. Lord, *Biophys Chem* **2010**, *152*, 15-20.

[6] a) T. Aida, E. W. Meijer, S. I. Stupp, *Science* **2012**, *335*, 813-817; b) L. L. Yang, X. X. Tan, Z. Q. Wang, X. Zhang, *Chem. Rev.* **2015**, *115*, 7196-7239.

[7] O. Goor, S. I. S. Hendrikse, P. Y. W. Dankers, E. W. Meijer, *Chem. Soc. Rev.* **2017**, *46*, 6621-6637.

[8] a) F. C. Mackintosh, J. Kas, P. A. Janmey, *Phys Rev Lett* **1995**, *75*, 4425-4428; b) P. R. Onck, T. Koeman, T. van Dillen, E. van der Giessen, *Phys Rev Lett* **2005**, *95*; c) J. S. Palmer, M. C. Boyce, *Acta Biomaterialia* **2008**, *4*, 597-612; d) Y. C. Lin, N. Y. Yao, C. P. Broedersz, H. Herrmann, F. C. MacKintosh, D. A. Weitz, *Phys Rev Lett* **2010**, *104*; e) C. P. Broedersz, F. C. MacKintosh, *Rev Mod Phys* **2014**, *86*, 995-1036; f) F. Meng, E. M. Terentjev, *Soft Matter* **2016**, *12*, 6749-6756.

[9] M. F. C. Romera, R. P. M. Lafleur, C. Guibert, I. K. Voets, C. Storm, R. P. Sijbesma, *Angew. Chem. Int. Edit.* **2017**, *56*, 8771-8775.

[10] P. H. Kouwer, M. Koepf, V. A. Le Sage, M. Jaspers, A. M. van Buul, Z. H. Eksteen-Akeroyd, T. Woltinge, E. Schwartz, H. J. Kitto, R. Hoogenboom, S. J. Picken, R. J. Nolte, E. Mendes, A. E. Rowan, *Nature* **2013**, *493*, 651-655.

[11] J. Boekhoven, J. M. Poolman, C. Maity, F. Li, L. van der Mee, C. B. Minkenberg, E. Mendes, J. H. van Esch, R. Eelkema, *Nat. Chem.* **2013**, *5*, 433-437.

[12] Y. Wang, M. Lovrak, Q. Liu, C. Maity, V. A. A. le Sage, X. Guo, R. Eelkema, J. H. van Esch, *J. Am. Chem. Soc.* **2019**, *141*, 2847-2851.

[13] Y. Wang, R. M. de Kruijff, M. Lovrak, X. Guo, R. Eelkema, J. H. van Esch, *Angew. Chem. Int. Edit.* **2019**, *58*, 3800-3803.

[14] Y. L. Yang, L. M. Leone, L. J. Kaufman, *Biophys J* **2009**, *97*, 2051-2060.

[15] a) T. Kato, *Science* **2002**, *295*, 2414-2418; b) A. Heeres, C. van der Pol, M. Stuart, A. Friggeri, B. L. Feringa, J. van Esch, *J. Am. Chem. Soc.* **2003**, *125*, 14252-14253; c) M. M. Safont-Sempere, G. Fernandez, F. Wurthner, *Chem. Rev.* **2011**, *111*, 5784-5814; d) V. M. P. Vieira, L. L. Hay, D. K. Smith, *Chem. Sci.* **2017**, *8*, 6981-6990; e) J. Raeburn, D. J. Adams, *Chem. Commun.* **2015**, *51*, 5170-5180.

[16] a) A. Brizard, M. Stuart, K. van Bommel, A. Friggeri, M. de Jong, J. van Esch, *Angew. Chem. Int. Edit.* **2008**, *47*, 2063-2066; b) J. Boekhoven, A. M. Brizard, M. C. A. Stuart, L. Florusse, G. Raffy, A. Del Guerzo, J. H. van Esch, *Chem. Sci.* **2016**, *7*, 6021-6031.

[17] F. Versluis, D. M. van Elsland, S. Mytnyk, D. L. Perrier, F. Trausel, J. M. Poolman, C. Maity, V. A. le Sage, S. I. van Kasteren, J. H. van Esch, R. Eelkema, *J. Am. Chem. Soc.* **2016**, *138*, 8670-8673.

[18] J. M. Poolman, C. Maity, J. Boekhoven, L. van der Mee, V. A. A. le Sage, G. J. M. Groenewold, S. I. van Kasteren, F. Versluis, J. H. van Esch, R. Eelkema, *J. Mater. Chem. B* **2016**, *4*, 852-858.