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Artificial Organelles: Reactions inside Protein–Polymer Supramolecular Assemblies

Martina Garni, Tomaž Einfalt, Mihai Lomora, Anja Car, Wolfgang Meier*, and Cornelia G. Palivan*

Abstract: Reactions inside confined compartments at the nanoscale represent an essential step in the development of complex multifunctional systems to serve as molecular factories. In this respect, the biomimetic approach of combining biomolecules (proteins, enzymes, mimics) with synthetic membranes is an elegant way to create functional nanoreactors, or even simple artificial organelles, that function inside cells after uptake. Functionality is provided by the specificity of the biomolecule(s), whilst the synthetic compartment provides mechanical stability and robustness. The availability of a large variety of biomolecules and synthetic membranes allows the properties and functionality of these reaction spaces to be tailored and adjusted for building complex self-organized systems as the basis for molecular factories.

Keywords: Amphiphilic copolymers · Artificial organelles · Enzymatic reactions · Nanoreactors

Introduction

Biological cells are complex structures with well-organized molecular machineries that work with high precision and efficiency.[1] In order to develop novel artificial molecular systems with similar complexity and multifunctionality, it is necessary to understand and mimic at the molecular level the various reactions involved in cell metabolism. Reactions inside the confined spaces of nanocompartments represent the core for the development of molecular factories for use in various domains, either in the biological environment (tissue, cells, *etc.*) or in environmental or technological applications. An essential step in the development of complex systems in terms of architecture and functionality is the bottom–up approach in which different modules are combined in a more complex topology. In this respect, knowledge of self-assembly acquired in biological systems is exploited in artificial architectures, because it is convenient for understanding conformational changes and functionality at the nanoscale, and serves for translational applications in the domains of medicine, catalysis and environment.[2,3] In particular, amphiphilic block copolymers are ideal candidates for designing complex systems, because they self-assemble in a variety of supramolecular 3D assemblies (micelles, nanoparticles, tubes, polymersomes) and planar membranes.[4,5] In addition to nanostructures, amphiphilic copolymers can also self-assemble in giant unilamellar vesicles (GUVs) as simple compartment models with μm-range sizes (Fig. 1).[6] However, control of self-assembly into the desired suprastructures is a challenging task. Their architecture and size strongly depend on the molecular properties of the block copolymer itself, such as hydrophilic-to-hydrophobic ratio, total molecular weight (M*w*) and polydispersity index (PDI),[7] and not all kinds of supramolecular nanostructures can be applied as platforms for developing confined reaction spaces at the nanoscale, such as nanoreactors, artificial organelles, or simple cell models. Polymersomes and GUVs are of particular interest in the design of compartmentalized reaction spaces, because their architectures mimic natural biocompartments (cells, organelles). In this respect, these vesicular structures are able to simultaneously host biomolecules (proteins, enzymes, nucleic acids) in their membranes, at their surfaces, and inside their inner cavities, and thus provide a protective space for complex reactions.[3] Compartmentalization is achieved by encapsulating active compounds during the self-assembly process of vesicle formation, whilst membrane proteins are inserted during or after vesicle formation. The mild conditions for vesicle formation are essential for preserving the structural integrity of biomolecule(s) and their activity. Compared to phospholipids which are the building block in cellular membranes, block copolymers have higher molecular weight and consequently form thicker and more stable membranes. *In situ* reactions in the cavities of polymersomes require rapid exchange of substrates/products with the environment, whilst keeping the active compounds protected inside. Thus, membrane permeability plays a pivotal role, and various methods have been reported to generate polymersomes with permeable membranes: i) synthesis of copolymers that intrinsically form porous membranes or membranes permeable to ions, *e.g.* specific oxygen species, ii) pore formation with chemical treatments, and iii) permeabilization of membranes by UV-irradiation.[8–11] A completely different strategy for permeabilizing synthetic membranes was developed in our group by applying a biomimetic approach in which membrane proteins or biopores are inserted into the synthetic membrane, and serve for passive or active transport of molecules through the membrane.[12] Reaction spaces based on protein–polymer assemblies are at the early stage of research, but we have already introduced models of synthetic functional systems that mimic artificial organelles, and these will be further developed and extended toward the design of artificial molecular factories. The complex scenario of requirements needed in molecular factories will be achieved by interfacing multiple reactions at the nanoscale in a hierarchical self-organization to provide the necessary flow of molecules/signals.

*Correspondence: Prof. W. Meier, Prof. C. G. Palivan
E-mail: wolfgang.meier@unibas.ch, cornelia.palivan@unibas.ch
University of Basel, Department of Chemistry
Klingelbergstrasse 80, CH-4056 Basel

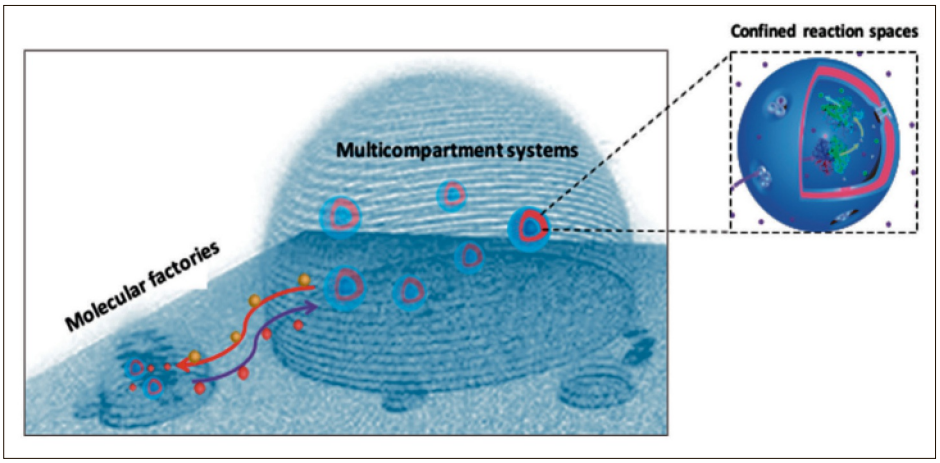

Fig. 1. Protein–polymer supramolecular assemblies and their use as reaction space at the nanoscale. Modified with permission from refs [3] and [13]. Copyright 2016 American Chemical Society.

Bioinspired Permeabilization of Polymer Membranes

Functional reconstitution of membrane proteins into synthetic membranes is complex, because they are normally denaturated in non-biological environments, and a key molecular factor is the membrane flexibility/fluidity for coping with the conformation and size of the membrane protein.[14] However, we have shown that poly(2-methyloxazoline)-*block*-poly(dimethylsiloxane) (PMOXA-PDMS) di- and tri-block copolymers generate polymersomes and GUVs by self-assembly under physiological buffer conditions with high flexibility and fluid membranes.[13] Although lateral diffusion properties of various fluorescent-labeled membrane proteins (AqpZ, OmpF and KcsA) within PMOXA-PDMS and PMOXA-PDMS-PMOXA GUV membranes indicated a significant hydrophobic mismatch between the membrane thickness (between 9 and 13 nm) and the size of the proteins (3.5–5 times), all these membrane proteins were successfully inserted and functional in the synthetic membranes (Fig. 2). The explanation for this interesting behavior for the membrane proteins in synthetic thick membranes is the high flexibility of the PDMS domain, which induces membrane fluidity similar to phospholipid bilayers.[15]

Reconstitution of membrane proteins/biopores to allow transport of ions/molecules is essential for reactions to be performed in confined nanospaces, such as nanoreactors and artificial organelles. In this respect, we were able to insert in a functional manner both biopores and membrane proteins in synthetic flexible membranes based on PMOXA-PDMS and PMOXA-PDMS-PMOXA amphiphilic copolymers. For example, we inserted ionomycin, a small ion transporter of 1.5 nm diameter in membranes 6 times thicker than its size. Interestingly, insertion of ionomycin rendered the polymersome permeable only to Ca^{2+} ions, and resulted in synthetic membranes with selective permeability that might be necessary for specific reactions *in situ* in polymersome cavities.[16] By successfully inserting the small pore forming peptide gramicidin (2.5 nm in length) into the membranes of polymersomes and GUVs, we engineered membranes selectively permeable to protons, Na^+ and K^+ ions (Fig. 3).[6] Indeed the outflux of protons from GUVs upon gA-insertion was proved by the change in the fluorescence of the inner cavity of the GUVs (Fig. 3, right).[6]

In order to allow transport of molecules, it is necessary to insert channel porins which allow diffusion of molecules with sizes smaller than the inner diameter of the pore. Outer membrane protein F (OmpF) was the first membrane protein we successfully reconstituted with full functionality into PMOXA-PDMS-PMOXA triblock copolymer membranes, and this allowed diffusion of molecules up to 600 Da.[17] In addition, we reconstituted FhuA, a channel protein with a large pore diameter of 39–46 Å elliptical cross-section, which ensured a rapid molecular flux into the cavity of PMOXA-PDMS-PMOXA polymersomes.[18]

A step further was realized by genetic or chemical modification of OmpF with specific molecules at key locations to influence the pore permeability, substrate selectivity and to induce membrane responsiveness. Such responsive synthetic membranes allow the design of nanoreactors with triggered activity that depends on the external environment, whilst preserving the vesicular architecture, and maintaining the encapsulated enzyme inside. (Fig. 4).[19,20] Such nanoreactors with triggered activity represent ideal candidates when a specific step in the flow of a molecular factory requires a reaction activated 'on demand' by the presence of a stimulus.

Interestingly, functional insertion of the membrane protein aquaporin (AqpZ), a highly selective water channel, allowed us to develop synthetic membranes with significantly higher water permeability than all commercial membranes.[21] Reconstitution of this membrane protein

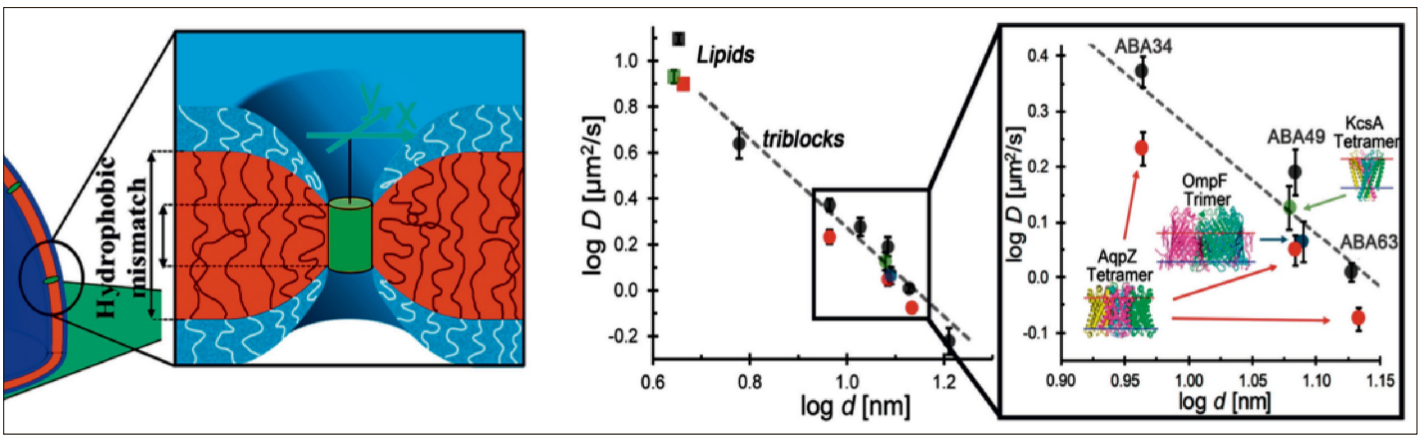

Fig. 2. Left: Schematic representation of the hydrophobic mismatch between membrane thickness and membrane proteins (represented as green cylinder). Right: Relationship between membrane thickness, *d*, and diffusion coefficient, *D*, of polymer chains and AqpZ, OmpF, KcsA membrane proteins within self-assembled membranes from ABA34, ABA49, and ABA63 triblock copolymers. Modified with permission from ref. [15]. Copyright 2015 American Chemical Society.

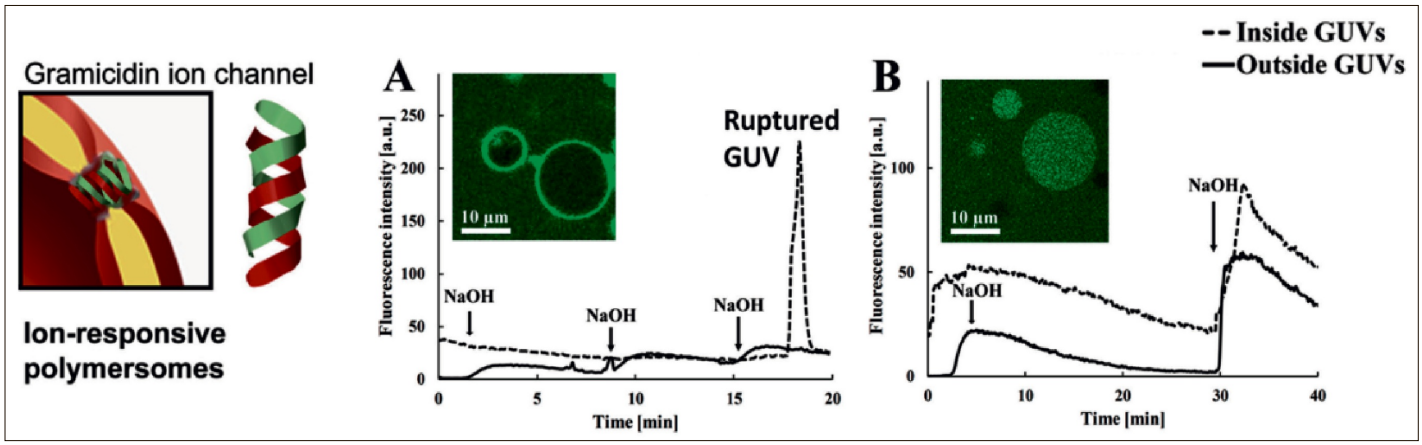

Fig. 3. Left: Design of polymersomes engineered for ion selective permeability based on gramicidin (gA) biopore insertion. Right: Change in fluorescence intensity inside GUVs between a non-permeabilized membrane (A) and a gramicidin permeabilized membrane (B). Modified with permission from ref. [6]. Copyright 2015 Elsevier.

creates an opportunity for developing biohybrid desalination membranes that are necessary for translational applications.

Nanocompartments as Reaction Spaces

The biomimetic approach for the formation of reaction spaces inside compartments (polymersomes and GUVs) involves encapsulation of desired active molecule(s) (biological or synthetic) together with membrane permeabilization by reconstitution of a membrane protein/biopore. Due to the sensitive nature of encapsulated/inserted biomolecules, it is essential to use relatively mild conditions, and to avoid organic solvents and high temperatures. One of the first examples of NRs was based on encapsulating β-lactamase in liposomes to hydrolyze '*in situ*' ampicillin to ampicillinoic acid, which was released from the nanoreactor through the OmpF pores. Interestingly, both OmpF and the encapsulated enzyme were not affected by introduction of methacrylate monomers into the liposome membrane to increase its stability by cross-linking polymerization.[17,22] A step further was realized when completely synthetic membranes based on PMOXA-PDMS-PMOXA copolymers were used to form a mechanically more stable, but still biocompatible, polymer compartment. The role of the polymersome is to simultaneously protect the encapsulated active compounds from environmental conditions (*e.g.* proteolytic attack), and allow them to act '*in situ*'. Polymer nanoreactors based on a single enzyme type have been developed with various proteins, such as horseradish peroxidase,[11,20] nucleoside hydrolase,[23] or acid phosphatase.[24] Interestingly, by encapsulating enzymes with dual-functions, such as haemoglobin, it was possible to design multifunctional nanoreactors to simultaneously transport oxygen and degrade peroxynitrites.[25]

Reactions inside nanocompartments should be chosen to effectively meet the challenges of the desired applications. For example, in the case of oxidative stress involving reactive oxygen species in high levels that can damage cells, it is essential to decrease their intracellular concentration. We introduced a novel approach to detoxify superoxide radicals by designing nanoreactors with superoxide dismutase (SOD) or its mimics inside the cavity of polymersomes equipped with OmpF.[26–29] Another interesting approach for providing appropriate conditions for the development of complex reaction systems, as in molecular factories, is to design stimuli-responsive nanoreactors, in which a reaction starts under specific conditions, for example a change in pH, or by irradiation. We developed a nanoreactor, which produces 'on demand' reactive oxygen species (ROS) based on encapsulation of an efficient photosensitizer, Rose Bengal–bovine serum albumin conjugate (RB–BSA) in the cavity of PMOXA-PDMS-PMOXA polymersomes. Such nanoreactors were able to produce ROS only when they were irradiated with the appropriate wavelength to induce singlet oxygen production by the photosensitizer.[30] A step further was achieved by simultaneously encapsulating a set of enzymes that work in tandem inside polymersomes: the close spatial proximity and precise positioning of the enzymes leads to a highly efficient cascade reaction. When a nanoreactor is uptaken and preserves its activity inside cells it can be considered as a simple artificial organelle that provides specific functionality to the cell.[31] We introduced the first artificial peroxisome by co-encapsulating superoxide dismutase and lactoperoxidase/catalase inside the cavity of polymersomes. Upon uptake by THP-1/HeLa cells, this simple peroxisome efficiently detoxified both O_2^- and H_2O_2.[32] Development of the artificial peroxisome using tandem enzymes found in the natural peroxisomes opens a

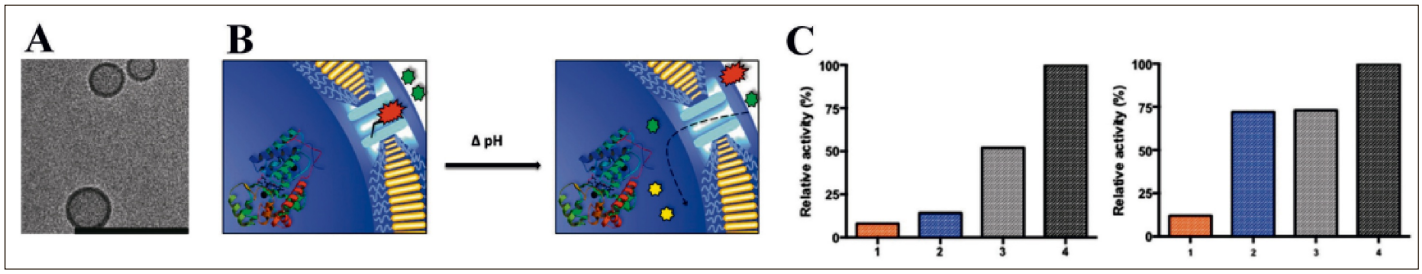

Fig. 4. A) Cryo-TEM of OmpF equipped PMOXA-PDMS-PMOXA nanoreactors. Scale bar 100 nm. B) Schematic representation of pH responsive OmpF. C) Substrate red conversion kinetics of nanoreactors equipped with different OmpFs: OmpF blocked with a pH responsive molecular cap (blue), OmpF with pH responsive molecular cap released (grey), Ompf-WT (black), and unpermeabilized nanoreactors (orange) at pH 5.5, at time 0 (left) and after 1 hour (right). Modified with permission from ref. [20]. Copyright 2015 American Chemical Society.

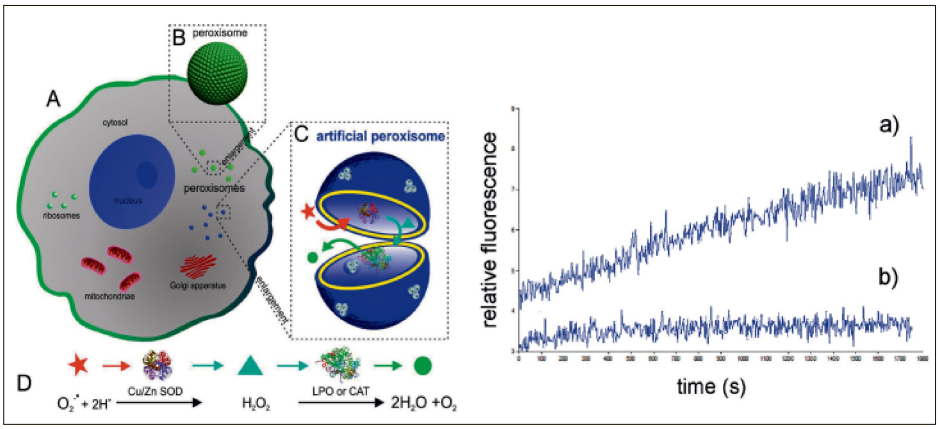

Fig. 5. Schematic drawing of a simplistic artificial peroxisome (AP) (left). ROS detoxification kinetics in cells under oxidative stress (right) without APs (a), and after incubation with APs (b). Modified with permission from ref. [31]. Copyright 2013 American Chemical Society.

new direction for complex multifunctional systems that can be regarded as 'cellular implants' (Fig. 5).[31] Despite the fact that nanoreactors and artificial organelles are in their early stage of research, they prove that nanoscience-based solutions are able to support the development of molecular factories. Once nanocompartments are engineered to perform complex functions, such as cascade reactions, energy conversion, signal transduction, minimal metabolism and synthesis of molecular compounds, they can be assembled into more elaborate systems that perform as basic molecular factories.

From Nanoreactors and Artificial Organelles toward more Complex Reaction Spaces

Synthetic compartments that combine polymer membranes with tailored permeability and catalytically active molecules represent essential building blocks for molecular factories. Molecular factories will involve interfacing different types of reaction spaces, one of them being presented here (polymersomes/GUVs) in a controlled manner to obtain the final product response. Additionally, the existence of a plethora of enzymes and enzyme mimics for encapsulation allows several different types of catalytic reaction (*e.g.* detoxification of reactive oxygen species, pro-drug activation, *etc.*), depending on the desired translational application. Further, polymersomes can be functionalized to expose at their external surfaces specific molecular moieties, which serve for targeting approaches, or surface immobilization: these exposed molecular entities will serve as points for specific interactions with biological systems (inter- or intra-cellular). In addition, such external molecular functionality will serve for zipping vesicles, which is an essential step for developing complex hierarchical systems based on self-organized architectures: more complex reactions will be possible in such complex reaction topologies. The first steps towards achieving such molecular complexity needed for developing nanoreactors and simple artificial organelles have been presented here. However, for the design of molecular factories there are still major challenges that have to be solved, such as controlling the flow of molecules between different reaction spaces, the interconnectivity of systems with complementary roles, and the overall architecture – multifunctionality of the final system. Last but not least, the expected high-added value of translational applications have to be considered and appropriately introduced into the complexity of the molecular factory design. Future challenges for the design of model artificial cells and complex molecular factories include the design of systems capable of division and reproduction, systems with metabolic activity switched on and off 'on demand', and systems that can synthesize or release naturally occurring substances.

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[1] S. M. Rafelski, W. F. Marshall, *Nature Rev. Mol. Cell Biol.* **2008**, *9*, 593.
[2] G. Gunkel-Grabole, S. Sigg, M. Lomora, S. Lörcher, C. G. Palivan, W. P. Meier, *Biomater. Sci.* **2015**, *3*, 25.
[3] C. G. Palivan, R. Goers, A. Najer, X. Zhang, A. Car, W. Meier, *Chem: Soc. Rev.* **2016**, *45*, 377.
[4] K. Letchford, H. Burt, *Eur. J. Pharma. Biopharma.* **2007**, *65*, 259.
[5] J. Kowal, X. Zhang, I. A. Dinu, C. G. Palivan, W. Meier, *ACS Macro Lett.* **2014**, *3*, 59.
[6] M. Lomora, M. Garni, F. Itel, P. Tanner, M. Spulber, C. G. Palivan, *Biomater.* **2015**, *53*, 406.
[7] D. E. Discher, A. Eisenberg, *Science* **2002**, *297*, 967.
[8] S. M. Kuiper, M. Nallani, D. M. Vriezema, J. J. Cornelissen, J. C. van Hest, R. J. Nolte, A. E. Rowan, *Org. Biomol. Chem.* **2008**, *6*, 4315.
[9] D. R. Arifin, A. F. Palmer, *Biomacromol.* **2005**, *6*, 2172.
[10] D. Grafe, J. Gaitzsch, D. Appelhans, B. Voit, *Nanoscale* **2014**, *6*, 10752.
[11] M. Spulber, A. Najer, K. Winkelbach, O. Glaied, M. Waser, U. Pieles, W. Meier, N. Bruns, *J. Am. Chem. Soc.* **2013**, *135*, 9204.
[12] C. Nardin, S. Thoeni, J. Widmer, M. Winterhalter, W. Meier, *Chem. Commun.* **2000**, 1433.
[13] F. Itel, M. Chami, A. Najer, S. Lörcher, D. Wu, I. A. Dinu, W. Meier, *Macromol.* **2014**, *47*, 7588.
[14] A. M. Seddon, P. Curnow, P. J. Booth, *Biochim. Biophys. Acta* **2004**, *1666*, 105.
[15] F. Itel, A. Najer, C. G. Palivan, W. Meier, *Nano Lett.* **2015**, *15*, 3871.
[16] M. Lomora, F. Itel, I. A. Dinu, C. G. Palivan, *PhysChemChemPhys* **2015**, *17*, 15538.
[17] C. Nardin, J. Widmer, M. Winterhalter, W. Meier, *Eur. Phys. J. E* **2001**, *4*, 403.
[18] M. Nallani, S. Benito, O. Onaca, A. Graff, M. Lindemann, M. Winterhalter, W. Meier, U. Schwaneberg, *J. Biotechnol.* **2006**, *123*, 50.
[19] S. Ihle, O. Onaca, P. Rigler, B. Hauer, F. Rodríguez-Ropero, M. Fioroni, U. Schwaneberg, *Soft Matter* **2011**, *7*, 532.
[20] T. Einfalt, R. Goers, I. A. Dinu, A. Najer, M. Spulber, O. Onaca-Fischer, C. G. Palivan, *Nano Lett.* **2015**, *15*, 7596.
[21] M. Kumar, M. Grzelakowski, J. Zilles, M. Clark, W. Meier, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 20719.
[22] A. Graff, M. Winterhalter, W. Meier, *Langmuir* **2001**, *17*, 919.
[23] A. Ranquin, W. Versées, W. Meier, J. Steyaert, P. Van Gelder, *Nano Lett.* **2005**, *5*, 2220.
[24] P. Broz, S. Driamov, J. Ziegler, N. Ben-Haim, S. Marsch, W. Meier, P. Hunziker, *Nano Lett.* **2006**, *6*, 2349.
[25] D. Dobrunz, A. C. Toma, P. Tanner, T. Pfohl, C. G. Palivan, *Langmuir* **2012**, *28*, 15889.
[26] F. Axthelm, O. Casse, W. H. Koppenol, T. Nauser, W. Meier, C. G. Palivan, *J. Phys. Chem. B* **2008**, *112*, 8211.
[27] V. Balasubramanian, O. Onaca, F. Axthelm, D. Hughes, M. Grzelakowski, W. Meier, C. G. Palivan, *J. Optoelectron. Adv. Mater.* **2009**, *1*, 1083.
[28] V. Balasubramanian, M. Ezhevskaya, H. Moons, M. Neuburger, C. Cristescu, S. Van Doorslaer, C. Palivan, *PhysChemChemPhys* **2009**, *11*, 6778.
[29] V. Balasubramanian, O. Onaca, M. Ezhevskaya, S. Van Doorslaer, B. Sivasankaran, C. G. Palivan, *Soft Matter* **2011**, *7*, 5595.
[30] P. Baumann, V. Balasubramanian, O. Onaca-Fischer, A. Sienkiewicz, C. G. Palivan, *Nanoscale* **2013**, *5*, 217.
[31] P. Tanner, V. Balasubramanian, C. G. Palivan, *Nano Lett.* **2013**, *13*, 2875.
[32] P. Tanner, O. Onaca, V. Balasubramanian, W. Meier, C. G. Palivan, *Chem. Eur. J.* **2011**, *17*, 4552.