Artificial Membranes

DOI: 10.1002/anie.201204645

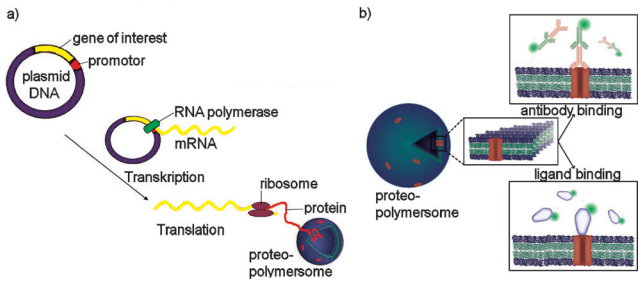
In Vitro Expressed GPCR Inserted in Polymersome Membranes for Ligand-Binding Studies**

Sylvia May, Mirjam Andreasson-Ochsner, Zhikang Fu, Ying Xiu Low, Darren Tan, Hans-Peter M. de Hoog, Sandra Ritz, Madhavan Nallani, and Eva-Kathrin Sinner**

G-protein-coupled receptors (GPCRs) constitute the largest gene family in the human genome (ca. 2% of all genes) and play an indispensable role in cell communication, cell adhesion, and signal transduction.[1] Given their central role in diverse physiological processes, it comes as no surprise that these seven-transmembrane domain proteins are significantly involved in many diseases and, indeed, about 40% of all marketed drugs or those in development target GPCRs.[2] For the majority of these receptors, however, the structure–function relationships remain elusive. Furthermore, there is a considerable number of orphan receptors with unknown endogenous ligands.[3] As such, substantial effort is directed towards the development of ligand-binding assays to identify either endogenous ligands or to screen for new drug leads.[4–9] A key determinant for the successful development of screening assays is the stable immobilization of GPCRs in an active conformation, preferably onto surfaces, to allow for a reliable micro-array-based screening format. Upon a screening hit such ligands can then be further tested in functional assays.

Conventional methods of producing GPCRs involve overexpression in host cells. This approach typically exerts a strain on the cells' metabolism, causing stress or even toxicity. Protein yields also tend to be low, owing to protein aggregation and mis-folding. An alternative is to extract GPCRs from the cell membrane, purify and then reconstitute them into lipid-based membrane systems. However, this approach often leads to the loss of functional integrity of the GPCR. Cell-free protein synthesis circumvents these pitfalls, but does not address the limited applicability of inherently fragile lipid bilayers.[10] To overcome the stability issues associated with lipid based systems, biomimetic membranes based on block-copolymers have been developed, which, owing to their amphiphilic nature, form bilayer membranes with lipid-like characteristics but with an enhanced stability.[11–14]

We previously described the in vitro synthesis of membrane proteins using block-copolymer membranes as an insertion platform.[15,16] Herein we show for the first time the incorporation of in vitro synthesized dopamine receptor D2 (DRD2; long form), a GPCR, into block copolymer vesicles, leading to so-called proteopolymersomes (Scheme 1). We use flow cytometry to demonstrate DRD2 expression, and investigate conformational integrity by antibody and ligand binding. The specificity of ligand binding is demonstrated by in vitro expression of DRD2 onto surface-immobilized polymersomes and displacement of bound fluorescent ligand from the receptor by unlabeled dopamine. This approach is of broad interest because, in principle, our system can be adapted to any type of membrane protein and may open new avenues for the development of ligand-binding assays in micro-array based formats.



Scheme 1. a) Synthesis of proteopolymersomes: The proteopolymersomes were produced by in vitro expression of membrane proteins and spontaneous insertion into polymer membranes. Complementary DNA (cDNA) encoding the protein and the polymersomes were directly added to the in vitro expression mixture. b) Antibody and ligand binding to proteopolymersomes. Upper box: The membrane proteins were detected in the purified proteopolymersomes through binding of a specific antibody and detection with a fluorescently labeled secondary antibody. Lower box: To determine if the membrane protein was incorporated into the polymersomes in an active conformation, ligand binding to the proteopolymersomes was carried out by incubating purified proteopolymersomes with fluorescently labeled dopamine.

[*] S. May, M. Andreasson-Ochsner, Z. Fu, Y. X. Low, Dr. D. Tan, Dr. M. Nallani
Patterning and Fabrication, Institute of Materials Research and Engineering, A*STAR (Agency for Science, Technology and Research
Research link 3, Singapore 117602 (Singapore)
E-mail: nallanim@imre.a-star.edu.sg
eva.sinner@boku.ac.at

Dr. H. M. de Hoog
Centre for Biomimetic Sensor Science, School of Materials Science and Engineering, Nanyang Technological University
50 Nanyang Drive, Singapore 637553 (Singapore)

Dr. S. Ritz
Physical Chemistry of Polymers
Max-Planck-Institut für Polymerforschung
Ackermannweg 10, 55128 Mainz (Germany)

Prof. Dr. E.-K. Sinner
University of Natural Resources and Life Science, BOKU, Vienna, Department of NanoBiotechnology
Muthgasse 11, 1190 Vienna (Austria)

[**] We thank Dr. Khiang Wee Lim, Director of the A*GA, A*STAR, Singapore for a grant for S.M. M. N. thanks A*STAR for financial support. E.-K. S. thanks Rain Fabricius-Brand for an introduction in interdisciplinary writing.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201204645.

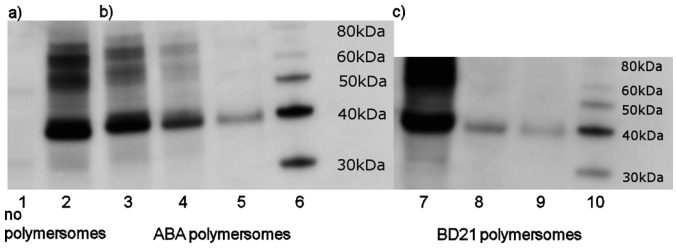


Figure 1. Western Blot of in vitro expressed DRD2 and subsequent purification of the polymersomes by centrifugal filtration. All expressed proteins were in solution when no polymersomes were used (lane 2). Each reaction containing DRD2 cDNA (lanes 2–5 and 7–9) showed distinct DRD2 bands corresponding to a size of 40 kDa. Upon centrifugal filtration the resuspended retentates (lanes 5 and 9) show a DRD2 band at 40 kDa, which corresponds to the protein that is associated to the polymersomes. Lane 1: no cDNA, no polymersomes added (negative control). Lane 2: DRD2 cDNA added, no polymersomes used, product was not purified. Lane 3: DRD2 cDNA added, ABA polymersomes used, product was not purified. Lane 4: Filtrate from lane 3 sample after centrifugal filtration. Lane 5: Resuspended retentate from lane 3 sample after centrifugal filtration. Lane 6: MagicMark XP Western Protein Standard. Lane 7: DRD2 cDNA added, BD21 polymersomes used, product was not purified. Lane 8: Filtrate from lane 7 sample after centrifugal filtration. Lane 9: Resuspended retentate from lane 7 sample after centrifugal filtration. Lane 10: MagicMark XP Western Protein Standard.

The cell-free in vitro protein expressions were supplemented with polymersomes formed from two different types of polymers: the triblock copolymer $PMOXA_{20}$-$PDMS_{54}$-$PMOXA_{20}$ and the diblock copolymer PBd_{22}-PEO_{13} (ABA and BD21, respectively, Figure 1). To separate the DRD2 proteopolymersomes from the components in the cell-free synthesis kit, the reaction mixtures were purified by centrifugal filtration using filters with 100 nm size cut-off. We then confirmed the cell-free expression of DRD2 in the presence of the polymersomes as well as that of pure DRD2 by Western blot analysis (Figure 1).

Each reaction mixture containing DRD2 cDNA resulted in a distinct band around 40 kDa, which was identified as DRD2 (actual molecular weight: 50 or 61 kDa). This altered migration (as compared to globular proteins) is typically observed for membrane proteins and was previously suggested to be correlated to the different stoichiometry of membrane protein–surfactant complex.[17] In our case the surfactant SDS might not have completely denatured the helical transmembrane regions, leading to a lower than expected binding and, thus, faster migration. The decreased intensity of the bands from the resuspended retentates suggests that around 25% of the expressed DRD2 was incorporated into both types of polymersomes (Figure 1, lanes 5 and 9). The remaining (75%) soluble DRD2 appeared in the filtrate (Figure 1, lanes 4 and 8).

To verify the association of DRD2 with the polymersomes, the resuspended retentates were detected by flow cytometry using DRD2 specific antibodies, and subsequent incubation with fluorescently labeled secondary antibodies (Figure 2). Only proteins incorporated into the polymersomes generated a signal because free proteins are out of the detection range owing to their small size. The significantly higher fluorescence signals of DRD2 functionalized ABA and BD21 proteopolymersomes compared to blank polymersomes (i.e., without cDNA; relative fluorescence intensity: 30–38%) indicates the incorporation of DRD2 into the polymersomes. To investigate the nature of the non-specific binding (NSB) of antibodies to unfunctionalized polymersomes, a second negative control was performed which involved polymersomes functionalized with a different membrane protein, that is, Claudin 2 (Cld2).[15] Its in vitro expression and incubation with the DRD2-specific antibody yielded NSB of 3% (data not shown). This result suggests that the NSB was mainly caused by interaction with the polymersome membrane, which is less accessible when membrane proteins are present.

Furthermore, the binding of antibodies to DRD2-functionalized polymersomes demonstrates that the N-terminus of the protein, which is the epitope of the antibody, was accessible to the antibodies, suggesting that a fraction of the DRD2 was incorporated into the polymersomes in the physiologically correct orientation, that is, with the C-terminus located in the "cytoplasm" and the N-terminus on the "extracellular side".[18]

The conformational integrity of polymersome incorporated DRD2 was further investigated by a ligand-binding assay using dansyl-dopamine as the ligand. The binding pocket for dopamine is formed by the seven transmembrane domains in DRD2 and thus dopamine can only bind to DRD2

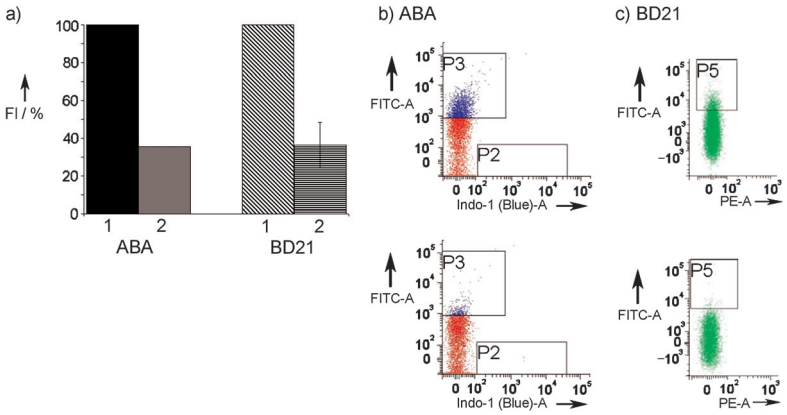


Figure 2. Detection of antibody binding to DRD2 proteopolymersomes and blank polymersomes by flow cytometry. Purified polymersomes and proteopolymersomes were incubated for 30 min with a specific antibody raised against DRD2. After rinsing, all samples were then incubated with a fluorescently labeled secondary antibody for 30 min and analyzed. a) Fluorescence intensities (FI) of the DRD2 proteopolymersomes with the captured fluorescently labeled secondary antibody. For clarity, all data are normalized against the FI of DRD2-functionalized proteopolymersomes (DRD2-ABA (1), and DRD2-BD21 (1)). The FI of blank ABA polymersomes (2) is 35% of total binding, while that of plain BD21 is 38% (2). b,c) Dot plots for the DRD2-ABA and DRD2-BD21 proteopolymersomes (upper plots) and pure ABA and BD21 polymersomes (lower plots), measured by flow cytometry. b) The reduced antibody binding to blank ABA polymersomes is clearly detected in gate P3. c) The reduced antibody binding to blank BD21 polymersomes is clearly detected in gate P5.

when it is present in the polymersomes in the correctly folded conformation and orientation.[18] Both polymersomes in solution and immobilized onto a surface were tested. For ligand-binding experiments in solution, the binding of fluorescent dansyl-dopamine to column-purified DRD2-functionalized BD21 proteopolymersomes was analyzed by flow cytometry.

For all ligand-binding studies a dansyl-dopamine concentration of 30 μM was chosen based on the recommended concentration by the supplier ($10 \times K_D$ of dopamine, where for the K_D we adopted the highest value reported in literature).[19] After incubation with 30 μM dansyl-dopamine for 30 min, higher fluorescence intensities were observed for DRD2 proteopolymersomes compared to the negative controls, confirming that DRD2 is able to bind fluorescently labeled dopamine ligand specifically, which indicates that the receptor is incorporated into the polymersomes in an active conformation (Figure 3).

Interestingly, in contrast to the antibody-binding experiments, virtually no NSB to the blank polymersomes was detected. The hydrophilic surface of the polymersomes apparently does not interact with the relatively hydrophobic dansyl-dopamine. In vitro expressed Cld2 polymersomes, however, yielded a NSB of 50% for dansyl-dopamine (Figure 3a). We postulate that in vitro expression causes NSB through hydrophobic interactions between dansyl-dopamine and the hydrophobic parts of the inserted membrane protein of interest, or through adsorbed residual proteins. The fact that the reverse was observed for antibody binding (i.e., relatively high NSB for pure polymersomes and low NSB for the Cld2 polymersomes) indicates that NSB for antibodies is dominated by electrostatic rather than hydrophobic interactions.

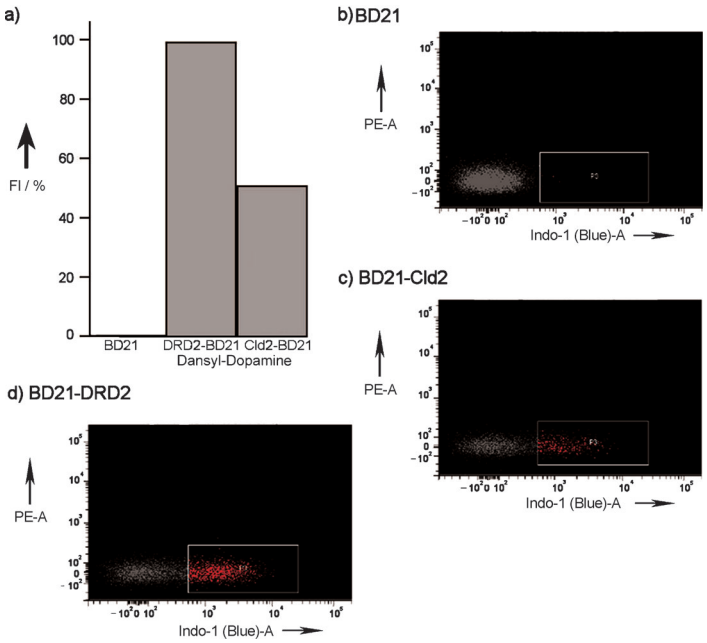


Figure 3. Detection of dansyl-dopamine binding to proteopolymersomes by flow cytometry. Purified proteopolymersomes were incubated with 30 μM dansyl-dopamine. a) The FI of the DRD2-proteopolymersomes (DRD2-BD21) is twice as high as the Cld2 negative control (Cld2-BD21, indicating BD21 proteopolymersomes containing the membrane protein Claudin 2) while virtually no binding of dansyl-dopamine was observed for blank BD21 polymersomes (BD21). The corresponding dot plots are shown for b) blank BD21 polymersomes, c) Cld2-BD21 proteopolymersomes, and d) DRD2-BD21 proteopolymersomes.

For future applications in biosensing or drug screening, it is of practical interest to immobilize the proteopolymersomes onto a surface. Therefore, we immobilized ABA polymersomes onto amino-functionalized glass chips in striped patterns (see Supporting Information for details). We chose ABA polymersomes because these can be conjugated in a specific and relatively straightforward manner by the use of tetrazoles.[20] DRD2 was then incorporated into these surface-patterned polymersomes by cell-free expression and subsequently incubated with 30 μM of dansyl-dopamine for 30 min. The resultant fluorescence pattern clearly revealed the binding of dansyl-dopamine to the DRD2 proteopolymersomes and indicated that a significant amount of DRD2 was present in its active conformation (Figure 4a). The low NSB (ca. 10%, Figure 4b and c) of the negative control (blank polymersomes) corroborates specific binding of dansyl-dopamine to DRD2-functionalized polymersomes.

The immobilized proteopolymersomes were then used to further characterize the specificity of ligand binding to the proteopolymersomes, by means of a replacement assay. In this case, the surface immobilized DRD2-proteopolymersomes were incubated with 30 μM dansyl-dopamine for 30 min. After rinsing, the chips were incubated for 30 min with solutions containing different concentrations of unlabeled dopamine (plain buffer, 1 μM, 10 μM, 100 μM, 1 mM, and 25 mM), and the fluorescence intensity before and after incubation was measured (Figure 4d). A decrease in fluorescence with increasing concentration of unlabeled dopamine was apparent, showing that dansyl-dopamine binds reversibly to the DRD2 proteopolymersomes and can be displaced by unlabeled dopamine. The resultant sigmoidal curve obtained by plotting fluorescence intensity against concentration is characteristic of specific ligand binding and we calculated a relative EC_{50} value of 30 μM.

With the experiments above, we have demonstrated the direct incorporation of a GPCR into polymer membranes using a cell-free in vitro synthesis method. Functional reconstitution of several types of membrane proteins, such as channel-forming proteins, rhodopsin, and ATP synthase, has been demonstrated by other groups despite the increased thickness of polymer membranes compared to lipid membranes, and we hypothesize that the incorporation of our GPCRs proceeds through a similar biophysical mechanism.[21–24] Simulations of the insertion of channel-forming proteins in polymer membranes suggests a compression of the highly flexible hydrophobic block of the membrane upon incorporation, which implies that the polymer membrane is able to adapt its local structure to the specific physical requirements of the membrane protein.[25] Together with the amphiphilicity of the membrane, this renders them amenable to the insertion of various classes of membrane proteins. We are currently optimizing our system to study how the

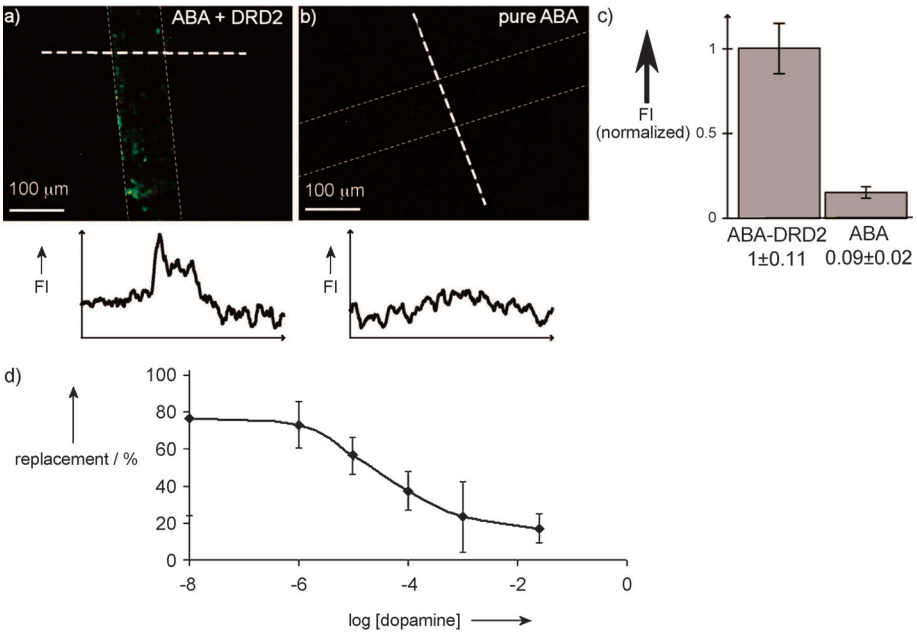


Figure 4. Dansyl-dopamine binding and replacement assay of DRD2-ABA polymersomes immobilized onto glass surfaces. a,b) Striped patterns of DRD2 proteopolymersomes and blank polymersomes. Dansyl-dopamine fluorescence was only detected within the stripes of DRD2 proteopolymersomes, indicating specific binding, whereas dansyl-dopamine fluorescence was much weaker for the blank polymersomes, indicating low NSB. The fluorescence profile along the dotted line is also shown. c) Comparison of fluorescence intensity (FI) of dansyl-dopamine in patterns containing DRD2 functionalized ABA or blank ABA (no cDNA). d) Replacement assay of dansyl-dopamine (30 µM) with unlabeled dopamine. The replacement shows a sigmoidal dependence on increasing unlabeled dopamine concentration.

membrane influences on the ligand-binding properties of the inserted GPCRs.

In conclusion, we have demonstrated the direct insertion of cell-free in vitro synthesized dopamine receptors into polymer membranes. Western blot analysis and flow cytometry measurements of antibody binding confirmed the association of the recombinant protein with the polymersomes. The demonstration of reversible ligand binding in solution and on a glass surface showed that the incorporated receptor was correctly folded and at least partially present in a physiological relevant orientation. This platform, in principle, allows the incorporation of any membrane protein for which the cDNA is available. Coupled with the chemical versatility and mechanical stability of polymersomes, our results underscore the technological potential of using polymersomes to develop reliable platforms for screening membrane proteins in a microarray format.

Experimental Section

For cell-free in vitro expression of DRD2 a TNT T7 coupled wheat germ extract system (Promega) was used. The method was performed as described elsewhere.[15] Proteopolymersomes obtained from the in vitro synthesis reaction were purified and used for subsequent experiments. Successful expression was determined by Western Blot analysis.

For antibody recognition, proteopolymersomes were labeled with a monoclonal primary antibody against DRD2 and a fluorescent secondary antibody was added and directly analyzed in the flow cytometer (λ_{ex} = 488 nm/(λ_{em}=505–550 nm). For ligand-binding experiments, purified DRD2 proteopolymersomes were passivated and subsequently incubated with 30 µM dansyl-dopamine ligand (FIVEphoton Biochemicals) for 30 min at 37 °C and directly analyzed by flow cytometry (λ_{ex} = 355 nm, λ_{em} = 505–550 nm) (see the Supporting Information for details).

For replacement assay, methacrylate functionalized ABA-polymersomes were covalently bound to the surface through a linker. In vitro synthesis was then performed on the patterned surface. After thorough rinsing, the chips were incubated with 30 µM dansyl-dopamine (30 min, dark, room temperature). The chips were rinsed again and imaged with an Olympus microscope (CKX41 with DP20 Digital Camera; magnification 10×; ISO 200, 2 s). Subsequent replacement was performed in the same manner with different concentrations of unlabeled dopamine (pure TMN buffer, 1 µM; 10 µM, 100 µM, 1 mM, and 25 mM). Binding data was analyzed using GraphPad Prism (see the Supporting Information for details).

Received: June 14, 2012
Revised: August 2, 2012
Published online: November 19, 2012

Keywords: biosensors · drug discovery · polymers · protein expression · receptors

[1] E. Jacoby, R. Bouhelal, M. Gerspacher, K. Seuwen, *ChemMedChem* **2006**, *1*, 760.
[2] M. A. Yildirim, K. I. Goh, M. E. Cusick, A. L. Barabasi, M. Vidal, *Nat. Biotechnol.* **2007**, *25*, 1119.
[3] X.-L. Tang, Y. Wang, D. L. Li, J. Luo, M. Y. Lui, *Acta Pharmacol. Sin.* **2012**, *33*, 363.
[4] Y. Hong, B. L. Webb, H. Su, E. J. Mozdy, Y. Fang, Q. Wu, L. Liu, J. Beck, A. M. Ferrie, S. Raghavan, J. Mauro, A. Carre, D. Mueller, F. Lai, B. Rasnow, M. Johnson, H. Min, J. Salon, J. Lahiri, *J. Am. Chem. Soc.* **2005**, *127*, 15350.
[5] C. Danelon, S. Terrettaz, O. Guenat, M. Koudelka, H. Vogel, *Methods* **2008**, *46*, 104.
[6] K. Bailey, M. Bally, W. Leifert, J. Voros, T. McMurchie, *Proteomics* **2009**, *9*, 2052.
[7] F. Degorce, A. Card, S. Soh, E. Trinquet, G. P. Knapik, B. Xie, *Curr. Chem. Genomics* **2009**, *3*, 22.
[8] R. Heilker, M. Wolff, C. S. Tautermann, M. Bieler, *Drug Discovery Today* **2009**, *14*, 231.
[9] M. Bally, K. Bailey, K. Sugihara, D. Grieshaber, J. Voros, B. Stadler, *Small* **2010**, *6*, 2481.
[10] F. Katzen, T. C. Peterson, W. Kudlicki, *Trends Biotechnol.* **2009**, *27*, 455.
[11] A. González-Pérez, K. B. Stibius, T. Vissing, C. H. Nielsen, O. G. Mouritsen, *Langmuir* **2009**, *25*, 10447.
[12] Z. Z. F. Meng, *J. Phys. Chem. Lett.* **2011**, *2*, 1533.
[13] A. González-Pérez, V. Castelletto, I. W. Hamley, P. Taboada, *Soft Matter* **2011**, *7*, 1129.

[14] D. Hua, L. Kuang, H. Liang, *J. Am. Chem. Soc.* **2011**, *133*, 2354.
[15] M. Nallani, M. Andreasson-Ochsner, C. W. Tan, E. K. Sinner, Y. Wisantoso, S. Geifman-Shochat, W. Hunziker, *Biointerphases* **2011**, *6*, 153.
[16] M. Andreasson-Ochsner, Z. Fu, S. May, L. Y. Xiu, M. Nallani, E. K. Sinner, *Langmuir* **2012**, *28*, 2044.
[17] A. Rath, M. Glibowicka, V. G. Nadeau, G. Chen, C. M. Deber, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 1760.
[18] C. Missale, S. R. Nash, S. W. Robinson, M. Jaber, M. G. Caron, *Physiol. Rev.* **1998**, *78*, 189.
[19] J. A. Gingrich, M. G. Caron, *Annu. Rev. Neurosci.* **1993**, *16*, 299
[20] H.-P. M. de Hoog, M. Nallani, B. Liedberg, *Polym. Chem.* **2012**, *3*, 302.
[21] C. Nardin, J. Widmer, M. Winterhalter, W. Meier, *Eur. Phys. J. E* **2001**, *4*, 403.
[22] H. J. Choi, C. D. Montemagno, *Nano Lett.* **2005**, *5*, 2538.
[23] C. Hyo-Jick, L. Hyeseung, D. M. Carlo, *Nanotechnology* **2005**, *16*, 1589.
[24] M. Kumar, M. Grzelakowski, J. Zilles, M. Clark, W. Meier, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 20719.
[25] G. Srinivas, D. E. Discher, M. L. Klein, *Nano Lett.* **2005**, *5*, 2343.
[26] H. Bermudez, A. K. Brannan, D. A. Hammer, F. S. Bates, D. E. Discher, *Macromolecules* **2002**, *35*, 8203.