Membrane Fusion Model

DOI: 10.1002/anie.201101951

Transmembrane Domain Peptide/Peptide Nucleic Acid Hybrid as a Model of a SNARE Protein in Vesicle Fusion**

*Antonina S. Lygina, Karsten Meyenberg, Reinhard Jahn, and Ulf Diederichsen**

Dedicated to Professor Gerhard Bringmann

Membrane fusion, one of the most fundamental processes in life, occurs when two separate lipid membranes merge into a single continuous bilayer.[1] This process is triggered by the specific action of fusion proteins like SNARE proteins (*s*oluble *N*-ethylmaleimide-sensitive factor *a*ttachment protein *re*ceptor) in the case of synaptic transmission.[2] In order to promote exocytotic fusion of synaptic vesicles in presynaptic nerve endings, a coiled-coil four-helix bundle is formed between two SNARE proteins residing in the plasma membrane (syntaxin-1A and SNAP-25) and a SNARE protein residing in the membrane of synaptic vesicles (synaptobrevin or VAMP), which brings the two merging membranes into close proximity.[1] However, the precise mechanism by which SNARE proteins execute the merger of the lipid bilayers is still unclear.[3] In particular, questions remain concerning the exact role of the transmembrane domains of synaptobrevin (Syb) and syntaxin-1A (Sx). It is not sufficient just to pin two bilayers together; an anchor such as a long lipid chain or a natural transmembrane domain (TMD) is required for effective fusion.[4] In the case of SUVs (*s*mall unilamellar *v*esicles) vesicle–vesicle fusion is induced in a sequence-specific manner already by a single TMD peptide of synaptic SNARE[5] and also by a G-protein of the vesicular stomatitis virus.[6,7] Furthermore, the crystal structure of the neuronal SNARE complex shows interactions between both the linkers and the TMDs in the fully assembled complex, supporting the view that these interactions promote the final steps of the fusion reaction.[8] To shed light on the role of membrane apposition and the transmembrane domains of fusion proteins, fusion experiments have been carried out using vesicles reconstituted with fusion proteins.[9] In addition, artificial model systems have been created that mimic the function of SNAREs in fusion reactions in vitro.[10–16] Artificial SNARE analogues have the advantage of simplifying the complex SNARE assembly reaction such that systematic structural variations and system compositions can be used to study the influence of each segment on the fusion process.

Herein we report on a novel SNARE-mimetic system consisting of hybrids between the TMD/linker segments from natural membrane-bound SNARE proteins and peptide nucleic acid (PNA) recognition motifs (Figure 1). With respect to the SNARE fusion mechanism, the TMD/PNA model systems can be used to define the orientation in which the recognition motifs dimerize; in this way, an artificial SNARE complex can be created that assembles either in a parallel or antiparallel orientation. Furthermore, we compared the efficiency of membrane fusion using identical (both derived from Sx) or different TMD units (derived from Syb and Sx). Finally, with the TMD/PNA SNARE analogue it is possible to capture the hemifusion state preceding the full membrane fusion.

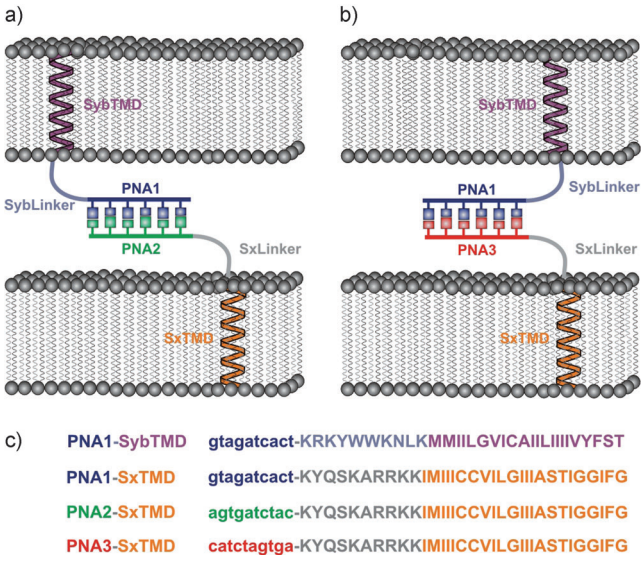

Figure 1. Simplified PNA/peptide model systems containing the native linker (light blue/gray) and TMD sequences of synaptobrevin (Syb, violet) and syntaxin-1A (Sx, orange). PNA oligomers were introduced as artificial recognition motifs providing a) a model with antiparallel (PNA1 (blue) with PNA2 (green)) orientation of the interacting strands and b) a model with parallel strand orientation (PNA1 (blue) with PNA3 (red)). c) Respective PNA/peptide sequences using the same color code for TMD, linker, and PNA units.

In the design of the novel SNARE-analogous TMD/PNA hybrid, the TMD/linker domain was based on the native

[*] A. S. Lygina, K. Meyenberg, Prof. Dr. U. Diederichsen
Institut für Organische und Biomolekulare Cehmie
Georg-August-Universität Göttingen
Tammannstrasse 2, 37077 Göttingen (Germany)
E-mail: udieder@gwdg.de
Prof. Dr. R. Jahn
Abteilung Neurobiologie
Max-Planck-Institut für Biophysikalische Chemie
Am Fassberg 11, 37077 Göttingen (Germany)

[**] We thank Heinrich Prinzhorn for DLS measurements. Generous support from the Deutsche Forschungsgemeinschaft DFG (SFB 803) is gratefully acknowledged.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201102628.

peptide sequences of Syb and Sx, whereas the SNARE recognition motif was replaced by PNA oligomers. PNA oligomers form stable and well-defined double-stranded nucleobase recognition complexes.[17] The PNA recognition motif resembles the double-stranded DNA already used in a SNARE analogue.[14] Nevertheless, PNA oligomers offer the advantage of the sequence-dependent formation of antiparallel as well as parallel duplexes with high thermal stability, sequence selectivity, an noncharged backbone, and protease resistance.[18] The peptide sequences for the peptide/PNA oligomers were synthesized by automated microwave-assisted solid-phase peptide synthesis (SPPS) for the peptide sequences following the 9-fluorenylmethoxycarbonyl (Fmoc) protocol, and manual SPPS was used for the extension of the oligomers with the PNA sequences (see the Supporting Information).

The sequences for the PNA recognition unit were chosen so that the duplexes form selectively in an antiparallel and a parallel manner with reasonable stability. PNA1 (gtagatcact) formed an antiparallel pairing complex with PNA2 (agtgatctac) with $T_m = 70\,^{\circ}C$ and a parallel duplex with PNA3 (catctagtga) with $T_m = 46\,^{\circ}C$ (for both measurements: 4 μM, 100 mM NaCl, 1 mM EDTA, 20 mM HEPES, pH 7.4; EDTA = ethylenediamine tetraacetic acid, HEPES = 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid).[17] The C-terminal extension of the PNA sequences with the linker peptides of Syb and Sx was shown by temperature-dependent UV spectroscopy to have only negligible influence on the thermal stability (see Figure S1 in the Supporting Information).

Fusion experiments were performed using vesicles with a hydrodynamic diameter of (100 ± 20) nm, as determined by dynamic light scattering (the preparation is described in the Supporting Information).[19] In the first assay the efficiency of lipid mixing was determined by using a standard dequenching assay based on fluorescence resonance energy transfer (FRET).[20] Vesicles containing PNA1-SybTMD were prepared with nitrobenzofuran (NBD) incorporated as a donor dye and lissamine rhodamine (Rh) as an acceptor dye, both of which were attached to the head group of the 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipid. Fusion with a vesicle that carries no fluorophore resulted in lipid mixing and an extension of donor–acceptor distance, as indicated by a lower FRET efficiency and increased donor emission (Figure 2a). Indeed, upon mixing of vesicles containing either PNA2-SxTMD or PNA3-SxTMD with vesicles containing PNA1-SybTMD (for conditions, see the Supporting Information), a significant increase in NBD emission was observed. The lipid mixing for PNA oligomers with a parallel orientation (PNA1/PNA3) was more efficient than that for antiparallel pairing PNA oligomers (PNA1/PNA2). The preference for the parallel arrangement of recognition units is in agreement with the orientation in the formation of the native SNARE complex. In a control experiment with vesicles containing PNA1-SxTMD, an increase in NBD emission was not observed, since PNA1 is not self-complementary with respect to duplex formation.

Bilayer fusion requires the merger of both the inner and the outer lipid leaflet of a membrane. In order to evaluate whether mixing of the inner leaflet takes place, the lipid-mixing experiments were repeated after treatment of the vesicle with sodium dithionite (Figure 2b). NBD fluorescence from the outer leaflet is selectively eliminated with sodium dithionite,[21] while emission from the inner leaflet remains

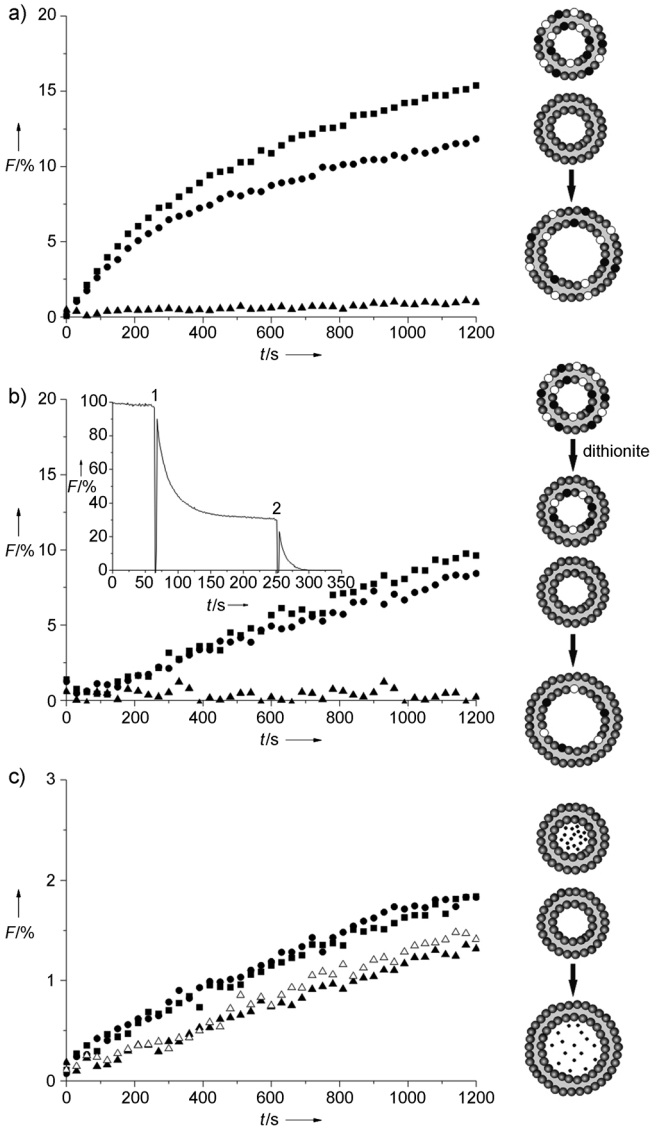

Figure 2. Lipid mixing in the outer and inner leaflets for vesicles containing TMD/PNA constructs. a) Total lipid mixing: NBD/Rh-labeled vesicles containing PNA1-SybTMD were mixed with unlabeled vesicles containing PNA1-SxTMD (control experiment, ▲), PNA2-SxTMD (antiparallel orientation, ●), and PNA3-SxTMD (parallel orientation, ■). b) Lipid mixing in the inner leaflet: NBD/Rh-labeled vesicles containing PNA1-SybTMD were treated with sodium dithionite and purified by size-exclusion chromatography before they were subjected to a lipid-mixing experiment like that described in (a). Inset: NBD intensity upon addition of sodium dithionite (1) and complete elimination of fluorescence after destruction of the vesicles with 0.1% TritonX-100 (2). c) Content mixing for vesicles containing TMD/PNA constructs: Vesicles containing PNA1-SybTMD with encapsulated SRB were mixed with unlabeled vesicles containing PNA1-SxTMD (control experiment, ▲), PNA2-SxTMD (antiparallel orientation, ●), PNA3-SxTMD (parallel orientation, ■), and with unlabeled vesicles without any constructs (leakage, △).

intact since sodium dithionite cannot penetrate the vesicle membrane. A calculated 56% overall decrease of NBD fluorescence for 100 nm vesicles was expected.[22] Indeed, in our system we measured a fluorescence decrease of about 60% upon addition of sodium dithionite (Figure 2b). Efficient lipid mixing, as indicated by FRET, was also observed for vesicles pretreated with sodium dithionite for PNA recognition motifs with both parallel and antiparallel orientations (Figure 2b). This FRET effect requires the participation of the NBD fluorophores in the inner leaflet and thus proves that not only the outer leaflets (hemifusion) but also the inner leaflets had merged (full fusion). However, notably the kinetics of total lipid mixing (Figure 2a) and lipid mixing in the inner leaflet (Figure 2b) are different. It is conceivable that in our model system the lipid mixing was at least partly terminated at the stage of hemifusion such that only the outer leaflets of the vesicles merged. Interestingly, the efficiency and kinetics of lipid mixing in the inner leaflet proved to be similar for both parallel and antiparallel orientations of PNA duplex.

In order to support our hypothesis of partial hemifusion, we performed content-mixing experiments (Figure 2c). The fluorescence self-quenching of sulforhodamine B (SRB) at high concentrations (>10 mM) was used in content-mixing experiments.[23] Two types of vesicles were prepared, one containing the encapsulated SRB and another one not labeled at all. Fusion of these vesicles was expected to lead to an increase in fluorescence caused by dilution of SRB (Figure 2c). Nevertheless, the efficiency of content mixing was determined to be very low, and no difference between parallel and antiparallel orientations of the PNA duplex was detected. Thus, based on both lipid-mixing and content-mixing experiments, it was concluded that synthesized TMD/PNA SNARE mimics lead to partial termination of the fusion process on the hemifusion stage.

The lipid-mixing experiments were further used to investigate the potential influence and participation of the TMDs in the fusion process. TMD/PNA-mediated vesicle fusion was examined by comparison of PNA recognition complexes with two identical (both Sx) and two different TMDs (Sx and Syb). If the TMD units contribute to the fusion process, a difference in the efficiency should be recognized. Lipid-mixing experiments were performed for the parallel (PNA1-SxTMD or PNA1-SybTMD with the complementary PNA3-SxTMD; Figure 3a) and the antiparallel recognition (PNA1-SxTMD or PNA1-SybTMD with the complementary PNA2-SxTMD; Figure 3b). Interestingly, for both orientations lipid mixing when identical Sx TMDs were employed was less efficient than with the natural Sx Syb TMD pair. Apparently, the TMD not only functions as a membrane anchor, but also contributes to the fusion process.

Addition of lysophosphatidylcholine (LPC) to the vesicle membrane leads to spontaneous positive membrane curvature, which inhibits the formation of the hemifusion intermediate, and therefore, prevents membrane fusion in cells and model systems.[16,24,25] In a control experiment, TMD/PNA-mediated vesicle fusion was carried out in the presence of 5 mol% LPC (Figure 3). Indeed, no lipid mixing was observed in the presence of LPC.

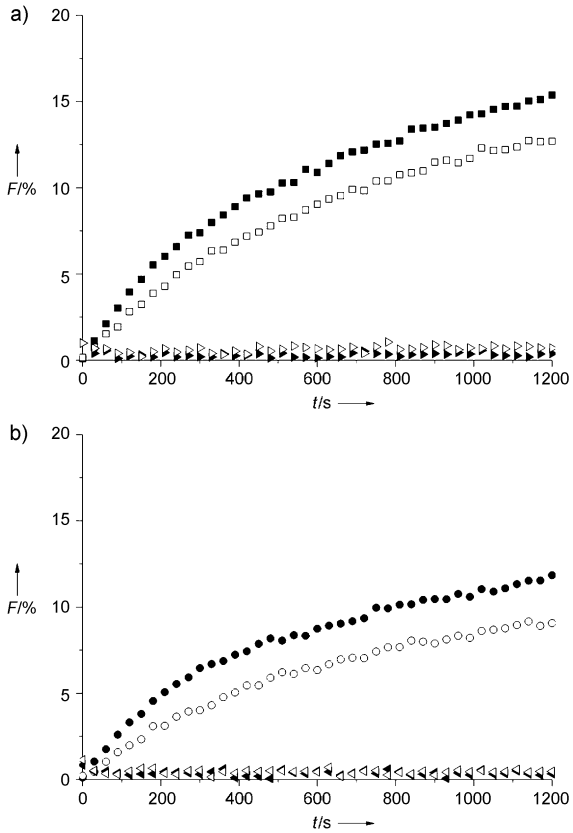

Figure 3. Total lipid mixing for vesicles containing PNA/peptide constructs. Unlabeled vesicles were mixed with NBD/Rh-labeled vesicles. a) Parallel orientation of PNA oligomers in PNA/peptide constructs: PNA3-SxTMD/PNA1-SybTMD (■), PNA3-SxTMD/PNA1-SybTMD (▶) after treatment of labeled vesicles with 5% egg lysophosphatidylcholine (LPC); PNA3-SxTMD/PNA1-SxTMD (□), PNA3-SxTMD/PNA1-SxTMD (▷) after treatment of labeled vesicles with 5% LPC; b) antiparallel orientation of PNA oligomers in PNA/peptide constructs: PNA2-SxTMD/PNA1-SybTMD (●), PNA2-SxTMD/PNA1-SybTMD (◀) after treatment of labeled vesicles with 5% LPC; PNA2-SxTMD/PNA1-SxTMD (○), PNA2-SxTMD/PNA1-SxTMD(◁) after treatment of labeled vesicles with 5% LPC. For details, see the Supporting Information.

The experiments described above show that both parallel and antiparallel strand alignment causes fusion, with the parallel orientation being somewhat more efficient. To shed further light on the role of strand recognition, we took advantage of the different melting temperatures of parallel and antiparallel strands.[17] Lipid-mixing experiments were conducted at various temperatures, spanning the range characteristic for PNA duplex formation to the formation of single strands (Figure 4). At 25°C, where PNA1/PNA2 and PNA1/PNA3 in both orientations form stable duplexes, we observed higher lipid-mixing efficiency for the parallel orientation, suggesting that this orientation is preferable for inducing vesicle fusion. The lipid-mixing efficiency for both types of PNA orientations becomes equal at 40°C, because close to the melting temperature of $T_m = 46$°C, the parallel-orientated PNA duplex (PNA1/PNA3) is partially unpaired; the stability of antiparallel-orientated PNA duplex (PNA1/PNA2) remains almost intact ($T_m = 70$°C). At 60°C, lipid mixing was detected only for the antiparallel orientation of

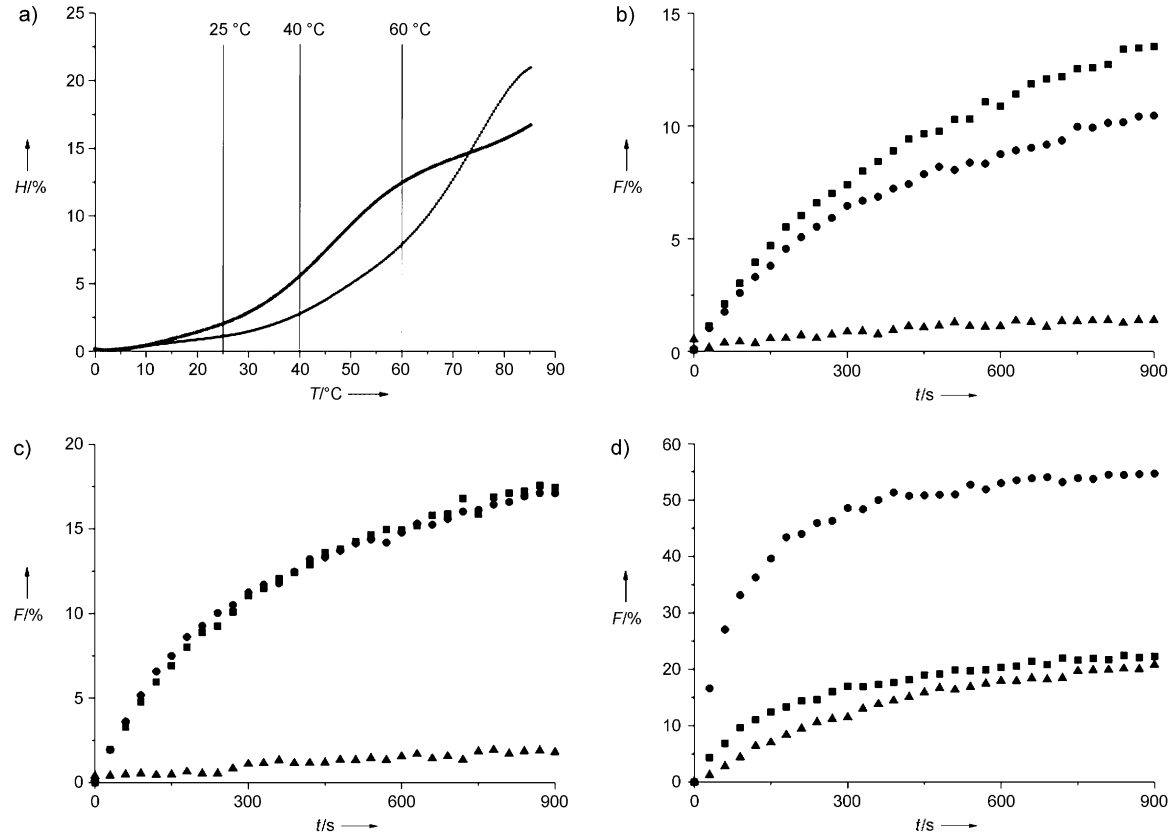

Figure 4. Temperature-dependent lipid-mixing experiments. a) Melting curves for PNA duplexes with parallel (PNA1/PNA3, black curve) and antiparallel (PNA1/PNA2, gray curve) orientations. NBD/Rh-labeled vesicles (PNA1-SybTMD) were mixed with unlabeled vesicles with PNA1-SxTMD (control experiment, ▲), PNA2-SxTMD (antiparallel orientation, ●), and PNA3-SxTMD (parallel orientation, ■). Lipid-mixing experiments were performed at b) 25, c) 40, and d) 60 °C. In general the lipid-mixing efficiency increases with higher temperature.[26,27]

PNA, while recognition with the potentially parallel PNA orientation was not possible and unspecific lipid mixing was observed. Thus, the lipid-mixing experiments carried out with PNA/peptide SNARE analogues at various temperatures are in good agreement with the stability of the corresponding PNA duplexes; fusion requires PNA–PNA recognition and can therefore be influenced by changing the temperature.

In conclusion, we have introduced a novel simplified model for membrane fusion mediated by SNARE proteins. For the transmembrane and linker domain the native peptide sequence of the two membrane-linked SNARE proteins was retained and they fused with the respective PNA recognition motif. Owing to the complementarity of the PNA base pairs, it was possible to investigate the fusion complexes with both helices linked on the same and opposite sides of the recognition motif. This system relies on only two strands instead of the four α helices required for functional SNARE complexes, which complicate the analysis of the final fusion step because of the formation of partial complexes.[2] The parallel SNARE-like orientation proved to be more effective for fusion. Overall, the new PNA/peptide hybrids facilitate fusion of vesicles dependent on the PNA base pair sequence and on temperature. In particular, the content-mixing fusion essay indicated the possibility of using the PNA/peptide hybrids to generate a significant amount of vesicles at the hemifusion stage. An interesting implication for understanding the mechanistic details of the SNARE fusion process was derived from the higher fusion efficiency of constructs with two different SNARE TMD units compared to that of PNA/peptide constructs in which the same TMDs are used in both constructs. Further insight in SNARE-mediated membrane fusion can be expected derived from the new PNA/peptide chimera; systematic modifications of the linker region, the TMD, and the recognition unit are currently under investigation.

Received: March 19, 2011
Published online: July 22, 2011

Keywords: hemifusion · membrane proteins · peptide nucleic acids · transmembrane domains · vesicles

[1] L. V. Chernomordik, M. M. Kozlov, *Nat. Struct. Mol. Biol.* **2008**, *15*, 675–683.
[2] R. Jahn, R. Scheller, *Nat. Rev.* **2006**, *7*, 631–643.
[3] A. T. Brunger, K. Weninger, M. Bowen, S. Chu, *Annu. Rev. Biochem.* **2009**, *78*, 903–928.
[4] J. A. McNew, T. Weber, F. Parlati, R. J. Johnston, T. J. Melia, T. H. Söllner, J. E. Rothman, *J. Cell Biol.* **2000**, *150*, 105–117.
[5] D. Langosch, M. J. Crane, B. Brosig, A. Hellwig, L. K. Tamm, J. Reed, *J. Mol. Biol.* **2001**, *311*, 709–721.

[6] D. Langosch, B. Brosig, R. Pipkorn, *J. Biol. Chem.* **2001**, *276*, 32016 – 32021.
[7] S. M. Dennison, N. Greenfield, J. Lenard, R. B. Lentz, *Biochemistry* **2002**, *41*, 14925 – 14934.
[8] A. Stein, G. Weber, M. C. Wahl, R. Jahn, *Nature* **2009**, *460*, 525 – 528.
[9] a) W. Wickner, R. Schekman, *Nat. Struct. Mol. Biol.* **2008**, *15*, 658 – 664; b) S. C. Harrison, *Nat. Struct. Mol. Biol.* **2008**, *15*, 690 – 698.
[10] A. Richard, V. Marchi-Artzner, M. N. Lalloz, M. J. Brienne, F. Artzner, T. Gulik-Krzywicki, M. A. Guedeau-Boudeville, J. M. Lehn, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 15279 – 15284.
[11] Y. Gong, Y. Luo, D. Bong, *J. Am. Chem. Soc.* **2006**, *128*, 14430 – 14431.
[12] M. Ma, A. Paredes, D. Bong, *J. Am. Chem. Soc.* **2008**, *130*, 14456 – 14458.
[13] M. Ma, Y. Gong, D. Bong, *J. Am. Chem. Soc.* **2009**, *131*, 16919 – 16926.
[14] G. Stengel, R. Zahn, F. Höök, *J. Am. Chem. Soc.* **2007**, *129*, 9584 – 9585.
[15] Y. H. M. Chan, B. van Lengerich, S. G. Boxer, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 979 – 984.
[16] H. R. Marsden, N. A. Elbers, P. H. H. Bomans, N. A. J. M. Sommerdijk, A. Kros, *Angew. Chem.* **2009**, *121*, 2366 – 2369; *Angew. Chem. Int. Ed.* **2009**, *48*, 2330 – 2333.
[17] P. Wittung, P. E. Nielsen, O. Buchardt, M. Egholm, B. Norden, *Nature* **1994**, *368*, 561 – 563.
[18] M. Eriksson, P. E. Nielsen, *Q. Rev. Biophys.* **1996**, *29*, 369 – 394.
[19] P. E. Schneggenburger, S. Müllar, B. Worbs, C. Steinem, U. Diederichsen, *J. Am. Chem. Soc.* **2010**, *132*, 8020 – 8028.
[20] D. K. Struck, D. Hoekstra, R. E. Pagano, *Biochemistry* **1981**, *20*, 4093 – 4099.
[21] J. C. McIntyre, R. G. Sleight, *Biochemistry* **1991**, *30*, 11819 – 11827.
[22] M. J. Hope, M. B. Bally, G. Webb, P. R. Cullis, *Biochim. Biophys. Acta Biomembr.* **1985**, *812*, 55 – 65.
[23] R. E. Jastimi, M. Lafleur, *Biospectroscopy* **1999**, *5*, 133 – 140.
[24] S. S. Vogel, E. A. Leikina, L. V. Chernomordik, *J. Biol. Chem.* **1993**, *268*, 25764 – 25768.
[25] P. L. Yeagle, F. T. Smith, J. E. Young, T. D. Flanagan, *Biochemistry* **1994**, *33*, 1820 – 1827.
[26] J. van der Bosch, C. Schudt, D. Pette, *Exp. Cell Res.* **1973**, *82*, 433 – 438.
[27] S. Zellmer, G. Cevc, P. Risse, *Biochim. Biophys. Acta Biomembr.* **1994**, *1196*, 101 – 113.

