Solid state NMR investigation of the interaction between biomimetic lipid bilayers and a de novo designed fusogenic peptide

Agrawal, P.R.; Kiinhe, S.R.; Hulsbergen, F.B.; Hofmann, M.; Langosh, D.; Groot, H.J.M. de; Hollander, J.

DOI: 10.1002/cbic.200600518

Solid State NMR Investigation of the Interaction between Biomimetic Lipid Bilayers and de novo Designed Fusogenic Peptides

Prashant Agrawal,[a] Suzanne Kiihne,[a] Johan Hollander,[a] Frans Hulsbergen,[a] Mathias Hofmann,[b] Dieter Langosch,[b] and Huub de Groot*[a]

Regulated fusion of biological membranes can be induced by membrane-associated peptides with low-complexity sequences.[1] Such peptides can drive fusion depending on their structural flexibility, but how the peptide changes the lipid phase is not known. Here we study the interaction of synthetic peptides with a biomimetic lipid mixture by using ^{31}P solid-state NMR spectroscopy.[2,3] We investigated two peptides: one previously shown to be rigid and virtually nonfusogenic, L16 ($K_3WL_{16}K_3$), and one that is flexible and highly fusogenic, LV16G8P9 (K_3WLVLVLVLGPVLVLVLVK_3).[1] A mixture of brain phosphatidylethanolamine (PE), brain phosphatidylserine (PS) and egg phosphatidylcholine (PC) at a 3:1:1 ratio was used to mimic biological membrane composition.[4] Here we show how the de novo designed peptides altered the phase behaviour of biomimetic lipid bilayers at a peptide to lipid ratio of 1:100. While L16 forms a rather stable α helix, LV16G8P9 can readily refold from α helix to β sheet and vice versa, by changing solvent polarity.[1]

Samples were prepared by dissolving 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE; Avanti Polar Lipids, AL, USA) in chloroform. Peptide dissolved in TFE was added at a peptide/lipid molar ratio of 0.01, as in previous fusion studies.[1] In the case of POPC lipid-bilayer studies, the peptide was dissolved in TFE, and was then mixed with POPC in chloroform at the desired peptide to lipid ratio. The mixtures were applied to 15 ultrathin cover-glass slides, dried with a stream of N_2 and kept in high vacuum, overnight. The plates were sprayed with fusion buffer (150 mM NaCl, 20 mM Tris-HCl, 0.2 mM EDTA, pH 7.4) and were equilibrated at 4 °C for 72 h at a relative humidity of 93% to yield samples with an estimated water content of ~0.2 μL mm^{-2}. Stacks of plates were sealed with Teflon tape and plastic wrap, inserted into a multichannel flat-coil probe and placed in a Bruker AV750 spectrometer. Proton-decoupled ^{31}P NMR spectra were recorded by using a Hahn-echo pulse sequence, with an echo delay of 40 μs and a recycle delay of 3 s.

The orientation of lipids can be probed by using ^{31}P NMR spectroscopy, since the observed line shape reflects the symmetry of the dynamic order of lipid molecules in a very characteristic manner.[5] The chemical shift anisotropy (CSA) tensor was partially averaged to an effective tensor that was axially symmetric and had principal axis values $\sigma_{\parallel} \sim 28$ ppm and $\sigma_{\perp} \sim -19$ ppm, for 0° orientation parallel to the rotation axis, and for 90° orientation perpendicular to the rotation axis, respectively. These values varied somewhat with lipid types and also depended on the exact orientation of the head-group with respect to the axis of rotation.[6] The isotropic ^{31}P chemical shifts of lipids in a buffer with pH 7.4 at 310 K were −2.7 ppm for POPC, −1.7 ppm for DOPE and −2.5 ppm for DOPS.

The ^{31}P NMR line shape of the pure lipid mixture was dominated by signals that are characteristic of oriented and cylindrical phospholipid phases (Figure 1 A).[2] Three narrow signals at 21.3, 22.5 and 32 ppm were assigned to 0° oriented PC, PE and PS in the bilayer phase, respectively, by comparison with values known for the pure lipids.[3,5,6] The broad signal at −20 ppm and the shoulder at −24 ppm revealed a cylindrical distribution of orientations in the unoriented phase, and the long axis of the cylinder was perpendicular to the field.[2] The other extremes of the cylindrical signals were hidden under the bilayer signals. When the unoriented response was simulated and combined with a set of narrow lines that represented the bilayer signal, a remarkably good reproduction of the experimental data was obtained (Figure 1 D). Given the overall sample composition of 60% PC, 20% PE and 20% PS, the simulated curves in Figure 1 indicated that ~64% of the ^{31}P signal accounted for cylindrical response at a ratio of ~36:13:15 (PC/PE/PS). The intensities of the narrow signals translated into relative fractions of ~24:7:5 (PC/PE/PS) for the aligned bilayer component (Figure 2).

Provided that the underlying model of aligned bilayers and cylindrical fractions was correct, the composition analysis was accurate to about 5%. The bilayer defects that formed increased-curvature domains were likely due to multilamellar bilayers and might have contained water in the centre.[2] These cylindrical fractions are expected to have relatively large diameters compared to the length scale of ~10 nm of lipid lateral diffusion.[2] Small cylinder shapes are unlikely since these would give rise to the narrowing of the ^{31}P CSA at a characteristic diffusion rate of ~10^{-11} m^2 s^{-1}.[2] Furthermore, an inverted hexagonal phase does not fit our data since its cylindrical rods have a diameter of only two lipid molecules, and lateral diffusion of lipids over the curved surface gives rise to partial averaging of the NMR anisotropy.[7] Also, unordered lipids would give rise to a narrow isotropic response due to rapid motional averaging.

Small vesicles composed of two differently shaped lipids, such as PC and PE, are known to exhibit strong compositional asymmetry between the two monolayers of a highly curved bilayer.[8] This asymmetry reduces the frustration between the

[a] P. Agrawal, Dr. S. Kiihne, J. Hollander, Dr. F. Hulsbergen, Prof. H. de Groot
Biophysical Organic Chemistry/Solid State NMR
Leiden Institute of Chemistry, Leiden University
Einsteinweg 55, 2333 CC Leiden (The Netherlands)
Fax: (+31) 71-5274603
E-mail: ssnmr@chem.leidenuniv.nl

[b] Dr. M. Hofmann, Prof. D. Langosch
Lehrstuhl Chemie der Biopolymere, Technische Universität München
Weihenstephaner Berg 3, 85354 Freising (Germany)

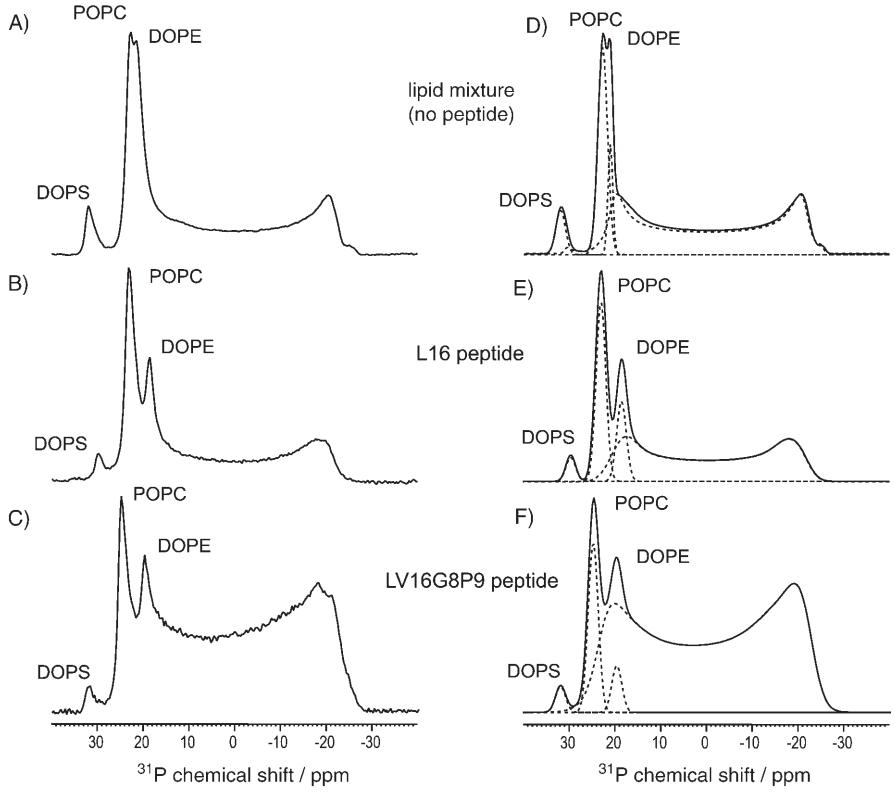

Figure 1. Proton-decoupled Hahn-echo ^{31}P NMRspectra of oriented bilayer samples; the bilayer normal was parallel to the magnetic field. Experimental spectra are shown in the left-hand panels (A, B and C). Simulated spectra are shown in the right-hand panels (D, E and F) and were generated by calculating the responses of various lipid species (dotted lines). A) and D) Lipid mixture without peptide; B) and E) lipid mixture loaded with nonfusogenic L16 peptide; C) and F) lipid mixture with fusogenic LV16G8P9 peptide.

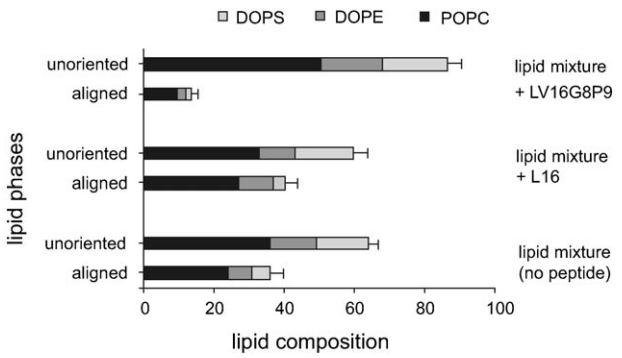

Figure 2. Comparison of the fraction of lipids present in the aligned and unoriented phases for a mixture that contained 60% POPC, 20% DOPS and 20% DOPE synthetic lipids. For the lipid mixture that contained the fusogenic peptide LV16G8P9 (1 mol%), the integrated intensities of the simulated ^{31}P signals in Figure 1 translated into composition ratios of ~10:2:2 (PC/PE/PS) and ~50:18:18 (PC/PE/PS) for the aligned-phase and unoriented-cylindrical response, respectively (upper panel). For the lipid mixture with the nonfusogenic L16 peptide (1 mol%) the aligned-bilayer component had a composition of ~27:10:3 (PC/PE/PS), and the cylindrical response had a composition of ~33:10:17 (PC/PE/PS; middle panel). For the lipid mixture without peptide the aligned-bilayer component had a composition of ~24:7:5 (PC/PE/PS) while the cylindrical-type response had a composition of ~36:13:15 (PC/PE/PS; lower panel). The error bars were estimated from the differences of the lipid compositions after analysis of two independently prepared samples.

two monolayers.[9] According to the analysis in Figure 2, the increased-curvature phase appears to contain less PC, while the flat membrane phase is PC enriched relative to the overall composition of 3:1:1 (PC/PE/PS). In the unoriented cylindrical fraction, the PE lipid molecules that prefer negative curvature might favour the inner layer, while PS lipids that prefer zero to positive curvature in the hydrated state could predominate in the outer layer. On the other hand, PC is thought to have neither a strong preference for the positively curved (outer) nor for the negatively curved (inner) monolayer.[10]

Upon incorporation of the nonfusogenic L16 peptide, which forms stable α helices,[1] the spectrum showed minor but reproducible changes (Figure 1B). The PC response shifted to 23.2 ppm and a PE peak was present at 18.6 ppm. In addition, the weak PS signal at 32 ppm in Figure 1A shifted to 29.5 ppm in Figure 1B. The broad signal that peaked at -18 ppm again reflects a cylindrical distribution in the increased-curvature phase and comprises ~60% of the lipids (Figures 1E and 2). The data can be simulated with a signal ratio of ~27:10:3 (PC/PE/PS) for the flat bilayer region and ~33:10:17 (PC/PE/PS) for the increased curvature region (Figures 1 and 2). Interestingly, when the lipid mixture contained the fusogenic peptide LV16G8P9, strikingly different results were obtained. The 0° oriented signal had a PC component that resonated with $\sigma_{\parallel}$ ~24.5 ppm, while the PE response was shifted to $\sigma_{\parallel}$ ~19.5 ppm. Simulations revealed that the increased-curvature phase had a significant fraction of vesicle-type signal (~16%). This was reflected by an asymmetric component in the signal, which perturbed the symmetric cylinder line shape. The asymmetric component could correspond to an increased fraction of end lipids that cover the hydrophobic edges of the bilayer. This suggests that cylindrical domains are short and more vesicle-like in the presence of the fusogenic peptide (Figure 1F). It is thus clear that LV16G8P9 stabilized the increased-curvature phase relative to the flat-bilayer phase. The data were simulated with relative intensities of ~10:2:2 (PC/PE/PS) for the flat-membrane fraction and ~50:18:18 (PC/PE/PS) for the increased-curvature component (Figures 1F and 2).

To test the effect of peptides on a POPC bilayer, which is the major component in the lipid mixture, we prepared POPC bilayers at a peptide/lipid molar ratio of 0.01. Figure 3 shows the

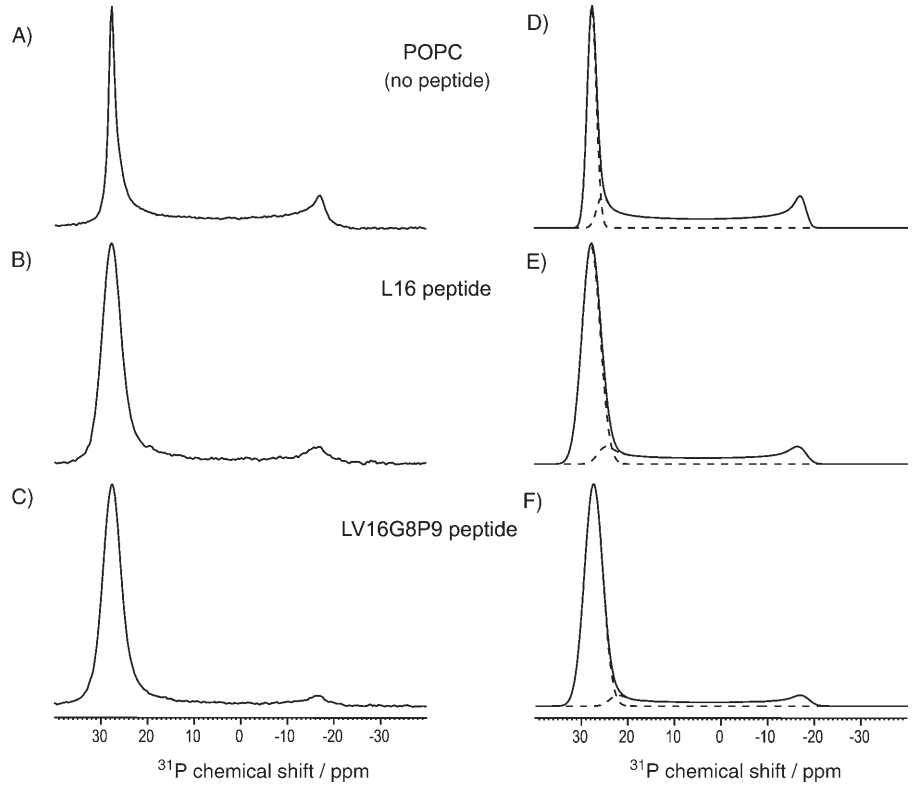

Figure 3. Proton-decoupled Hahn-echo ^{31}P NMR spectra of oriented POPC; the bilayer normal was parallel to the magnetic field (left-hand panels). A) POPC without peptide; B) POPC loaded with nonfusogenic L16 peptide (1%); C) POPC with fusogenic LV16G8P9 peptide (1%). D), E) and F) Simulated spectra generated by calculating the responses of various lipid line shapes (dotted lines).

^{31}P NMR spectra of POPC bilayers with and without peptides. In the absence of peptide, the POPC bilayers were aligned and exhibited a 0° oriented signal at 27 ppm and a broad signal at −18 ppm. The simulated curves in Figure 3D indicate that ~50% of the ^{31}P signal accounts for cylindrical response. Addition of nonfusogenic peptide, L16, moderately increased the level of order in the POPC bilayer, in which the aligned component represented ~70% of the lipids (Figure 3B and E). After addition of the fusogenic peptide, LV16G8P9, the POPC bilayers were aligned and the cylindrical response accounted for only ~20% of lipids (Figure 3C and F). Figure 4 shows the ^{31}P NMR spectra of POPC bilayers with 1–4% LV16G8P9. The addition of LV16G8P9 moderately increased the cylindrical response. With 4% LV16G8P9 the spectrum was mainly a cylindrical-shaped line; this accounted for ~80% of the lipids (Figure 4C and F). The signal at the 0° peak was broader as compared to the POPC membrane; this indicates the wobble of the POPC lipid molecules.

Thus, it appears that the fusogenic peptide, LV16G8P9, strongly induced curvature relative to either the pure or L16-containing lipid mixtures. Obviously the fusogenic peptide can induce excess membrane curvature provided that the bilayer contains PE and/or PS in addition to POPC. Our data thus indicate that synergy between the fusogenic peptide and lipid component(s) is required if a phase change is to occur. In particular, the cone-shaped PE is known to favour membrane fusion, which is attributed to its ability to support negative-bilayer curvature in fusion intermediates.[11] Both PE and PS have indeed been shown to enhance liposome fusion that is driven by peptides that correspond to soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) transmembrane domains.[12] Recently, enhanced stabilization of cylinder-type structures has also been observed for other membrane-active peptides. For example, antimicrobial peptides induce cylinder-phase formation upon interaction with a two-component POPC–POPG model system.[2] Peptide-induced lipid tubule formation has been reported for a de novo designed 18-residue amphipathic helical peptide[13] and for gramicidin D.[14] In addition, the unoriented phase of the sample with LV16G8P9 also contained less PC than the aligned phase, but the ratio of ~50:18:18 (PC/PE/PS) appeared somewhat closer to 3:1:1 than for the pure lipid system.

The membrane-fusogenic and structurally unstable LV16G8P9 might promote positive-membrane curvature by intercalating into the lipid head-group region of the outer layer. In line with the analysis of the pure lipid system, positive curvature of the outer layer of the cylindrical bilayer could be accommodated by redistribution of PE into the negatively curved inner monolayer. Circular dichroism measurements indeed indicate that LV16G8P9 exists mainly as β-sheets and -turns in liposomes (B. Poschner and D.L., unpublished results). This is consistent with the induction of curvature by peripheral association with the outer layer. This situation is reminiscent of amphipathic fusion peptides of viral fusogens. These peptides are thought to catalyze membrane fusion by disturbing lipid bilayer structure and inducing curvature by peripheral intercalation with lipids.[15] In particular, the fusion peptide of influenza haemagglutinin has been shown to associate laterally with membranes in a mixture of conformations, β sheet as well as α helix.[16,17] It can assume a boomerang shape that is thought to be critical for fusion initiation.[16] Finally, molecular dynamics simulations have provided evidence that fusion peptides can promote formation of a convex bulge, which might facilitate close initial contact of hydrated repulsive membranes and promote exchange of lipids at an early stage of fusion.[18] Hence, the LV16G8P9 peptide could induce membrane fusion by induction of local positive curvature as proposed for natural viral-fusion peptides.

In our model system, the water fraction in the sample is too small to stabilize intact vesicles, which can undergo fusion.[9]

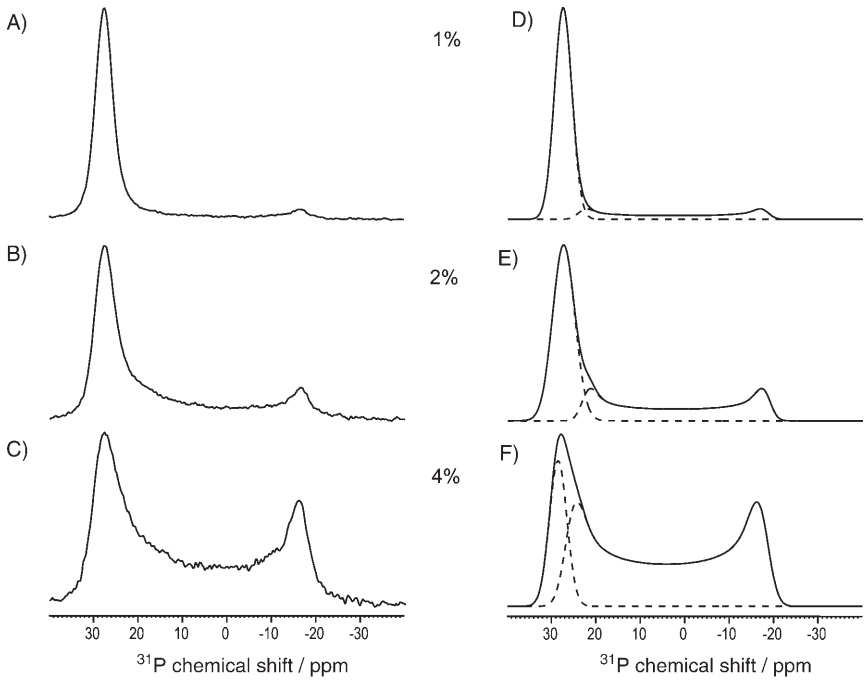

Figure 4. Proton-decoupled Hahn-echo ^{31}P NMR spectra of oriented POPC bilayers with fusogenic peptide LV16G8P9. The bilayer normal was parallel to the magnetic field (panels A, B and C). The simulated spectra are shown in the right-hand panels, and were generated by calculating the responses of various CSA patterns for lipid phases (dotted lines). A) POPC loaded with 1% fusogenic LV16G8P9 peptide. The integrated intensities of the simulated ^{31}P signals in panel D translate into ~80% for the aligned phase and ~20% for the unoriented cylindrical response. B) POPC with 2% nonfusogenic LV16G8P9 peptide. The aligned lipid phase was ~60%, and the unoriented cylindrical response was ~20% (panel E). C) POPC with 4% fusogenic LV16G8P9 peptide. The simulated ^{31}P signals in panel F translate into ~20% for the aligned phase and ~80% for the unoriented cylindrical response.

Yet the anisotropic NMR response revealed increased curvature at low water content, which is considered a prerequisite for fusion.[19,20] During membrane fusion lipids go through various phases, and lipidic rearrangement proceeds to the formation of hemifusion, which precedes complete fusion.[21] How exactly the fusogenic peptide does this is a matter for further detailed investigation.

In summary, solid-state NMR studies on mechanically aligned bilayers made up of a biomimetic three-lipid mixture show that strong perturbations of the lipid bilayer can be induced by a small fraction of a synthetic peptide. Considering the complexity of a three-lipid system and the role of peptides in interacting with the inner and outer monolayers of the membrane, these experimental data are of surprisingly high quality and can be analyzed in a consistent fashion. The data lead to the conclusion that small changes in the peptide content or character can cause significant and cooperative changes in membrane conformation; this is consistent with the current understanding of peptide-regulated fusion events. The data suggest that fusion induced by LV16G8P9 involves a curved structure that corresponds to the unoriented component seen here. Our observations can be reconciled with stabilization of positive curvature in the outer layer by intercalation of peptide. Furthermore, peptide-induced positive curvature might be balanced by redistribution of the negative-curvature agent, PE, into the inner monolayer.

Acknowledgements

Financial support for this work is received from the Volkswagenstiftung Conformational Control of Biomolecular Function Funding Initiative. The 750 MHz equipment was financed in part by demonstration project BIO4-CT97-2101 of the commission of the European Communities.

Keywords: lipids · membranes · NMR spectroscopy · peptides · solid-state NMR

[1] M. Hofmann, K. Weise, J. Ollesch, P. Agrawal, H. Stalz, W. Stelzer, F. Hulsbergen, H. de Groot, K. Gerwert, J. Reed, D. Langosch, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14776 – 14781.
[2] J. J. Buffy, M. J. McCormick, S. Wi, A. Waring, R. I. Lehrer, M. Hong, *Biochemistry* **2004**, *43*, 9800 – 9812.
[3] K. J. Hallock, D. K. Lee, A. Ramamoorthy, *Biophys. J.* **2003**, *84*, 3052 – 3060.
[4] J. W. Deutsch, R. B. Kelly, *Biochemistry* **1981**, *20*, 378 – 385.
[5] K. A. H. Wildman, D. K. Lee, A. Ramamoorthy, *Biochemistry* **2003**, *42*, 6545 – 6558.
[6] J. M. Pearce, R. A. Komoroski, *Magn. Reson. Med.* **1993**, *29*, 724 – 731.
[7] F. Picard, M. J. Paquet, E. J. Dufourc, M. Auger, *Biophys. J.* **1998**, *74*, 857 – 868.
[8] R. Lipowsky, *Curr. Opin. Struct. Biol.* **1995**, *5*, 531 – 540.
[9] S. A. Safran, P. A. Pincus, D. Andelman, F. C. Mackintosh, *Phys. Rev. A* **1991**, *43*, 1071 – 1078.
[10] J. A. Killian, M. R. R. De Planque, P. C. A. Van Der Wei, I. Salemink, B. De Kniijff, D. V. Greathonse, R. E. Koeppe II, *Pure Appl. Chem.* **1998**, *70*, 75 – 82.
[11] L. Chernomordik, A. Chanturiya, J. Green, J. Zimmerberg, *Biophys. J.* **1995**, *69*, 922 – 929.
[12] D. Langosch, J. M. Crane, B. Brosig, A. Hellwig, L. K. Tamm, J. Reed, *J. Mol. Biol.* **2001**, *311*, 709 – 721.
[13] S. Lee, T. Furuya, T. Kiyota, N. Takami, K. Murata, Y. Niidome, D. E. Bredesen, H. M. Ellerby, G. Sugihara, *J. Biol. Chem.* **2001**, *276*, 41224 – 41228.
[14] F. Moll, 3rd, T. A. Cross, *Biophys. J.* **1990**, *57*, 351 – 362.
[15] C. M. Wasniewski, P. D. Parkanzky, M. L. Bodner, D. P. Weliky, *Chem. Phys. Lipids* **2004**, *132*, 89 – 100.
[16] Y. Li, X. Han, A. L. Lai, J. H. Bushweller, D. S. Cafiso, L. K. Tamm, *J. Virol.* **2005**, *79*, 12065 – 12076.
[17] Q. Huang, C. L. Chen, A. Herrmann, *Biophys. J.* **200**4, 87, 14 – 22.
[18] P. Lague, B. Roux, R. W. Pastor, *J. Mol. Biol.* **2005**, *354*, 1129 – 1141.
[19] L. Yang, H. W. Huang, *Science* **2002**, *297*, 1877 – 1879.
[20] L. Yang, L. Ding, H. W. Huang, *Biochemistry* **2003**, *42*, 6631 – 6635.
[21] D. P. Siegel, *Biophys. J.* **1999**, *76*, 291 – 313.

Received: December 4, 2006
Published online on February 27, 2007

