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^{13}C and ^{15}N NMR evidence for peripheral intercalation of uniformly labeled fusogenic peptides incorporated in a biomimetic membrane

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Abstract

Membrane fusion requires drastic and transient changes of bilayer curvature and here we have studied the interaction of three de novo designed synthetic hydrophobic peptides with a biomimetic three-lipid mixture by solid state NMR. An experimental approach is presented for screening of peptide–lipid interactions and their aggregation, and their embedding in a biomimetic membrane system using established proton-decoupled ^{13}C, ^{15}N and proton spin diffusion heteronuclear $^{1}H-^{13}C$ correlation NMR methods at high magnetic field. Experiments are presented for a set of de-novo designed fusion peptides in interaction with their lipid environment. The data provide additional support for the transmembrane model for the least fusogenic peptide, L16, while the peripheral intercalation model is preferred for the fusogenic peptides LV16 and LV16G8P9. This contributes to converging evidence that peripheral intercalation is both necessary and sufficient to trigger the fusion process for a lipid mixture close to a critical point for phase separation across the bilayer.

Keywords: Oriented bilayer; Transmembrane membrane fusogenic polypeptide; Membrane fusion; Solid state NMR

1. Introduction

Biological membrane fusion requires drastic restructuring of lipid bilayers mediated by fusogenic membrane proteins, such as SNAREs and viral fusion proteins. Membrane fusion is thought to proceed via stalk formation and hemifusion, which leads to fusion pore formation that allows for complete fusion. Fusion peptides of viral fusogens are thought to catalyze membrane fusion by disturbing the lipid bilayer structure and inducing curvature by peripheral intercalation with lipids [1]. Previous work has indicated that low complexity peptides designed to mimic the characteristics of natural fusion domains can indeed show fusogenicity, which correlates with their structural plasticity [2]. These peptides can drive the fusion of liposome models for vesicles containing PC, PE and PS in a 3:1:1 ratio [3]. At these concentrations the lipid system is subject to physical frustration and sufficiently close to a critical point for phase separation to induce cooperativity and control over the suprastructure by a very small amount of peptide, ~1%. Recent data suggest that fusion involves stabilization of positive curvature in the outer layer by an intercalating peptide mediating stalk formation, which is generally considered an important intermediate step for fusion. This peptide-induced positive curvature in the outer layer may be accommodated by redistribution of the negative curvature-agent, PE, into the inner layer of the liposome, thereby stabilizing the curved bilayer structure relative to the planar bilayer [4].

Here we perform a set of solid-state NMR assays on a series of uniformly labeled fusogenic peptides. The data contribute to converging evidence that the more fusogenic peptides are peripherally intercalated at the membrane–water interface. Proton-decoupled ^{13}C, ^{15}N and proton spin diffusion heteronuclear $^{1}H-^{13}C$ correlation NMR experiments are used to characterize the secondary structure and orientation of the peptides in some detail. The ^{15}N line shape is sensitive to the orientation of the peptide and ^{13}C and heteronuclear experiments provide information about the secondary structure and location of peptides in vesicles. In particular proton spin diffusion is a good method to study membrane protein topology

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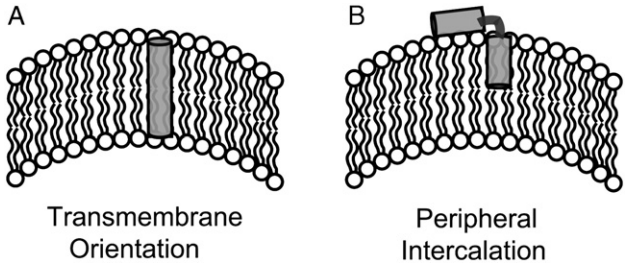

Fig. 1. Schematic representation of possible orientations of peptides in lipid bilayers. In case of transmembrane orientation (A) the peptide is buried in the bilayer while in case of peripheral intercalation (B) the peptide is partially inside the bilayer as well as partially interacting with the lipid head groups.

of non-oriented bilayer samples [5,6]. In the spin-diffusion approach, dipolar couplings between protons mediate a transfer of nuclear polarization from well-defined sources. The magnetization is selected based on the mobility or other spin properties and is allowed to transfer to the desired targets in the peptide [5]. Since the dipolar coupling is distance dependent, the rate of transfer is determined by the proximity of the sink spins to the source spins and this provides insight into how peptides can intercalate with the lipid membrane [5]. The liquid-crystalline nature of the lipid bilayer leads to high-resolution Magic Angle Spinning (MAS) ^{1}H and ^{13}C spectra [5].

Proton-decoupled ^{15}N solid-state NMR spectroscopy is another established method to provide information on the alignment of peptides within oriented membrane samples in a straightforward manner [7–11]. To perform solid-state ^{15}N NMR structural analyses, oriented lipid bilayer samples loaded with peptides can be introduced into the magnet of the NMR spectrometer with the bilayer normal parallel to the magnetic field direction. When ^{15}N solid-state NMR measurements are performed on monomeric polypeptides reconstituted into oriented lipid bilayers, rapid lateral and rotational diffusion averages the shift anisotropy [7]. The resulting solid-state NMR signal is a direct probe of the extent of molecular alignment relative to the bilayer normal/B_0-vector and the ^{15}N chemical shift is a good indicator of the orientation of polypeptides [12]. Different sites within a molecule can be distinguished based on the anisotropy of the ^{15}N chemical shift and this can be used to obtain orientational constraints for the molecule [7]. In addition, tilting, wobbling and vibrational motions, conformational changes, or quenching of rotation or diffusion can give incomplete averaging of the anisotropy and leads to broad NMR signals, providing information about the structural variability of the system [13].

The studies in this article focus on the behavior of three peptides, one previously shown to be rigid and virtually non-fusogenic, L16, one flexible and fusogenic, LV16, and a third that is highly fusogenic, LV16G8P9 [3]. According to early investigations, the predominant phospholipids in synaptic vesicles, i.e. PC (~50%), PE (~30%) and PS (~10%), comprise ~90% of all lipids [14]. We have performed control experiments, where we have mixed 60% PC, 20% PE and 20% PS from natural sources and found that head-group composition is more important for fusogenicity than acyl chain identity since replacement of the natural acyl chain mixture by the synthetic ones did not have a significant effect [15]. This suggests that mimicking head groups, rather than acyl chains, is most important for modeling the natural system. Hence a mixture of phosphatidylethanolamine (PE), phospatidylserine (PS), and phosphatidylcholine (PC) in a 3:1:1 ratio is used here to simulate the biological membrane composition of synaptic vesicles [14]. The behavior of the peptides is studied in biomimetic lipid bilayers at peptide to lipid ratios of 1:100 using different solid-state NMR techniques.

L16: K K K W L L L L L L L L L L L L L L K K K
LV16: K K K W L V L V L V L V L V L V L V K K K
LV16G8P9: K K K W L V L V L V L G P V L V L V K K K

The two possible topological models given in Fig. 1 have been used in the past to describe the lipid disturbing behavior of fusion peptides. For instance, the fusion peptide of influenza hemagglutinin has been shown to associate laterally with membranes in a mixture of conformations, β-sheet as well as α-helix. It can assume a boomerang-shape that is thought to be critical for fusion initiation. This leads to the peripheral intercalation model of Fig. 1B, where the peptide lies on the bilayers and interacts with the lipid head groups, perpendicular to the bilayer normal. In comparison, in the transmembrane model peptides are oriented approximately parallel to the bilayer normal, where Lys are buried in the hydrophobic interior while the middle hydrophobic core is close to the membrane–water interface (Fig. 1A) [16].

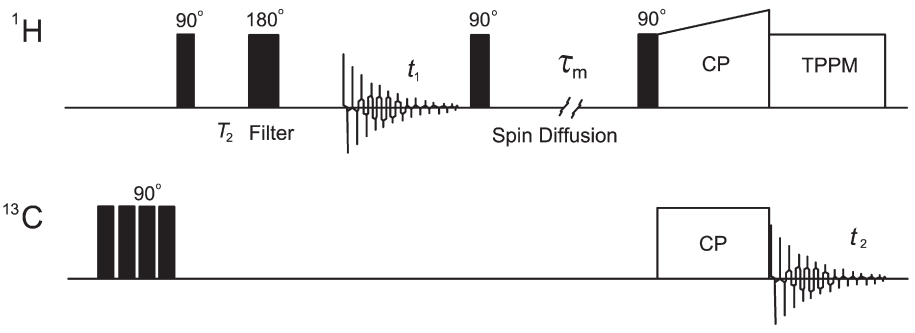

Fig. 2. Pulse sequence for the 2-D MAS heteronuclear correlation experiment with proton spin diffusion and ^{13}C detection. CP: cross-polarization, TPPM: two pulse phase modulation decoupling.

2. Materials and methods

POPC, DOPS and DOPE were purchased from Avanti Polar Lipids, Birmingham, AL. Peptides were synthesized and purified according to the procedures reported in [17]. Concentrations of peptide solutions were determined from the tryptophan absorbance at 294 nm in a 1:1 (v/v) mixture of TFE and dimethyl sulfoxide using an extinction coefficient of 5600 M^{-1} cm^{-1}. Liposomes were prepared from lipid mixtures at a weight ratio POPC: DOPE:DOPS=3:1:1 in chloroform with peptides which were first dissolved in trifluoroethanol (TFE). The mixture is dried under a stream of nitrogen to a thin film and rehydrated with a fusion buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 5 mM DTT). Complete removal of TFE from the sample was checked with ^{1}H NMR. Liposomes were formed after vortexing and freeze thawing the mixture, followed by bath sonication for 60 min while cooling with ice. The sample was centrifuged at 16,000×*g* for 20 min to remove lipid aggregates. The membrane-bound peptide sample was then transferred to a 4-mm NMR rotor. The oriented lipid bilayer samples are prepared as described in [4].

Experiments on lyophilized peptides were performed using a Bruker AV-750 MHz spectrometer, equipped with a double channel MAS probe head. Peptides (5 mg) in 50% acetic acid (vol/vol) were frozen in liquid nitrogen, lyophilized, and transferred into a 4-mm MAS rotor. 1D CP/MAS NMR spectra were recorded at room temperature with a ^{13}C radio frequency of 188 MHz and using a MAS frequency of 12.5 kHz. The ^{13}C-carbonyl resonance of U-[^{13}C,^{15}N]-Tyr–HCl at 172.1 ppm was used as an external reference for the calibration of the isotropic ^{13}C chemical shifts. The 1D ^{13}C and ^{1}H NMR spectra of peptides in vesicles were recorded at 277 K using CP/MAS with a spinning frequency of 7 kHz. The ^{13}C-carbonyl resonance of U-[^{13}C,^{15}N]-Tyr–HCl at 172.1 ppm and the ^{1}H resonance of H_2O at 4.8 ppm were used as an external reference for the calibration of the isotropic ^{13}C and ^{1}H chemical shifts, respectively.

The pulse sequence for the 2-D ^{1}H spin-diffusion experiment with ^{13}C detection is shown in Fig. 2 [5]. At the start of the sequence, four 90° pulses on the ^{13}C channel destroy residual ^{13}C magnetization. After an initial 90° pulse on the ^{1}H channel, ^{1}H magnetization from the mobile lipid segments and H_2O is selected by a T_2 filter, while the ^{1}H signals of the more rigid peptide relax due to the strong ^{1}H–^{1}H dipolar couplings. The 180° pulse in the middle of the T_2 filter refocuses the isotropic chemical shift evolution and the effect of the B_0 field

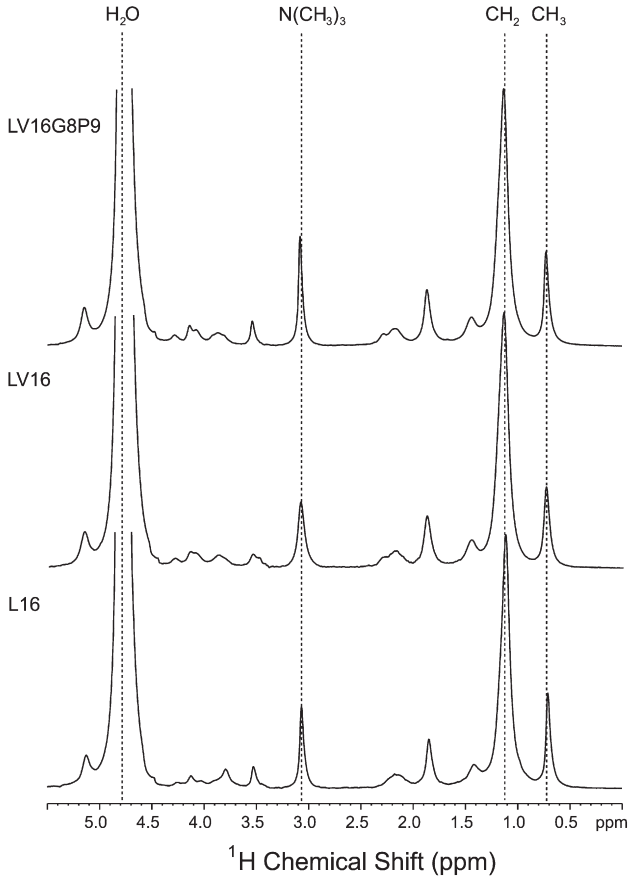

Fig. 4. One-dimensional ^{1}H MAS NMR spectra of biomimetic lipid mixtures loaded with uniformly labeled peptides, L16, LV16 and LV16G8P9. The data were recorded at 277 K with a spinning frequency of 7 kHz.

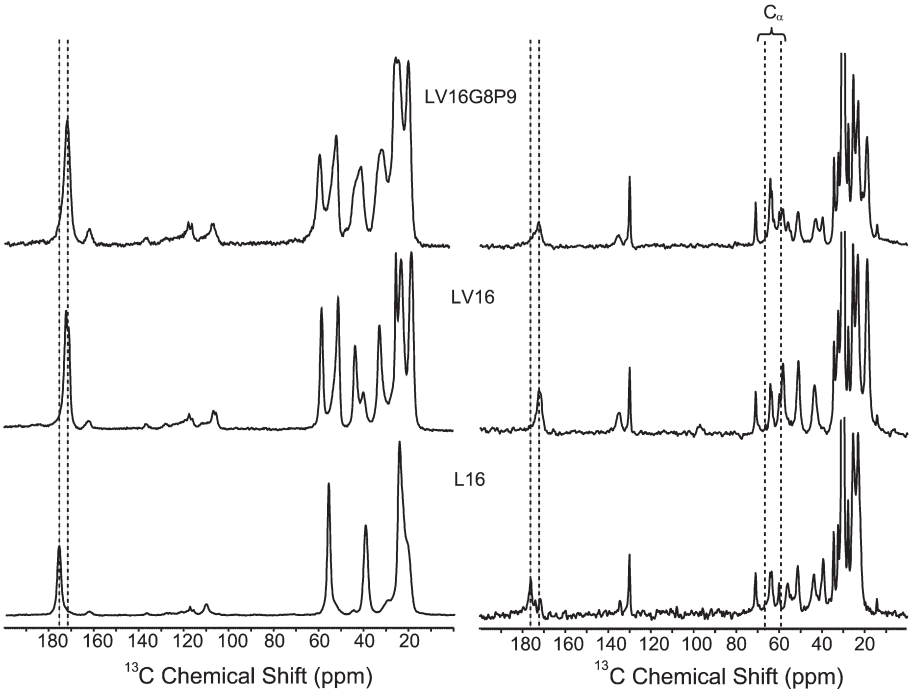

Fig. 3. One-dimensional ^{13}C-natural abundance CP-MAS solid-state NMR spectra of lyophilized peptides illustrating the differences in the ^{13}C-carbonyl resonances for L16, LV16 and LV16G8P9 (Left panel). One-dimensional ^{13}C CP-MAS solid-state NMR spectra of uniformly labeled peptides in a biomimetic lipid mixture (Right panel). The signals between 20 ppm and 60 ppm correspond to Cα, Cβ, as well as side-chain carbons.

inhomogeneity. Next, the lipid ^{1}H magnetization evolves under the isotropic chemical shift interaction for the t_1 period. A ^{1}H 90° pulse then stores the magnetization along the z-axis and allows it to exchange via spin diffusion for a mixing period, τ_m. After the mixing time, the ^{1}H magnetization is rotated to the CP effective field direction by $\pi/2$ and locked. A simultaneous CP pulse on the ^{13}C-channel with ramped field strengths transfers the ^{1}H magnetization to ^{13}C-spins, which are then detected during t_2.

The 2-D ^{1}H proton driven spin-diffusion (PDSD) experiments were performed with ^{1}H spin diffusion mixing times between 50 ms and 400 ms and spinning frequencies of 7 kHz, regulated to ±2 Hz by a pneumatic control unit. The data were acquired using a standard cross polarization pulse sequence for the transfer of magnetization between ^{1}H and ^{13}C [5]. The CP contact time was 512 μs. A Goldman–Shen T_2 filter (1.96 ms) was applied to selectively excite the more mobile lipid protons [6]. Experiments were run for different spin diffusion mixing times, 50 ms$<\tau_m<$400 ms. The data were collected in 640 scans with a spectral width of 94 kHz. T_1 values of different lipid protons were measured in a standard inversion-recovery experiment. All PDSD measurements were performed at a temperature of 277 K. The lower temperature was chosen to optimize the signal to noise ratio.

Oriented samples were inserted into a multichannel flat coil probe head and placed in a Bruker AV750 spectrometer with the membrane normal oriented parallel or perpendicular to the magnetic field direction. Proton-decoupled 1D ^{15}N solid-state NMR spectra were recorded using a CP Hahn echo pulse sequence with a 90° pulse length of 7.8 μs, an echo delay of 40 μs, 25 K scans of 1 K data points, and a recycle delay of 3 s (Fig. 8) [18]. A line broadening of 10 Hz was applied before Fourier transformation. The spectra were processed without first order phase correction and calibrated to NH_4Cl at 41.5 ppm chemical shift. The NMR measurements were performed using similar conditions of pH 7.4, at a temperature of 310 K and with ~30 min of equilibration time in the spectrometer before recording the data. Spectra were collected over 3 hr intervals to monitor changes.

For the quantitative analysis of 2-D spin-diffusion spectra, ^{1}H cross sections were extracted from series of 2-D spectra with different mixing times. The peak intensities were determined relative to the signal strengths for the first spectrum in the series, collected with the shortest mixing time of 50 ms. Peak intensities were corrected for T_1 relaxation during the mixing time by multiplication with $\exp(\tau_m/T_1)$ and plotted against $\sqrt{\tau_m}$ (Fig. 9) [5,6]. Error bars in Fig. 9 represent the noise level at the respective signals in the 2-D spectra.

3. Results and discussion

The orientational topology and secondary structure of membrane-associated peptides is an important aspect of their structure–function relationship. To assess whether the fusogenicity of the peptides is related to their structural properties, the secondary structure was studied under different conditions. First, to determine how the peptides fold when all solvent is removed, high-resolution ^{13}C 1D solid-state NMR data were collected from lyophilized samples, which is the starting material for the fusion assays (Fig. 3). The ^{13}C-carbonyl chemical shift at ~175 ppm is characteristic for an α-helix, while the signal at ~170 ppm is generally attributed to β-sheet [19]. Hence, L16 with its carbonyls resonating around 175.8 ppm predominantly forms α-helices. In contrast, LV16

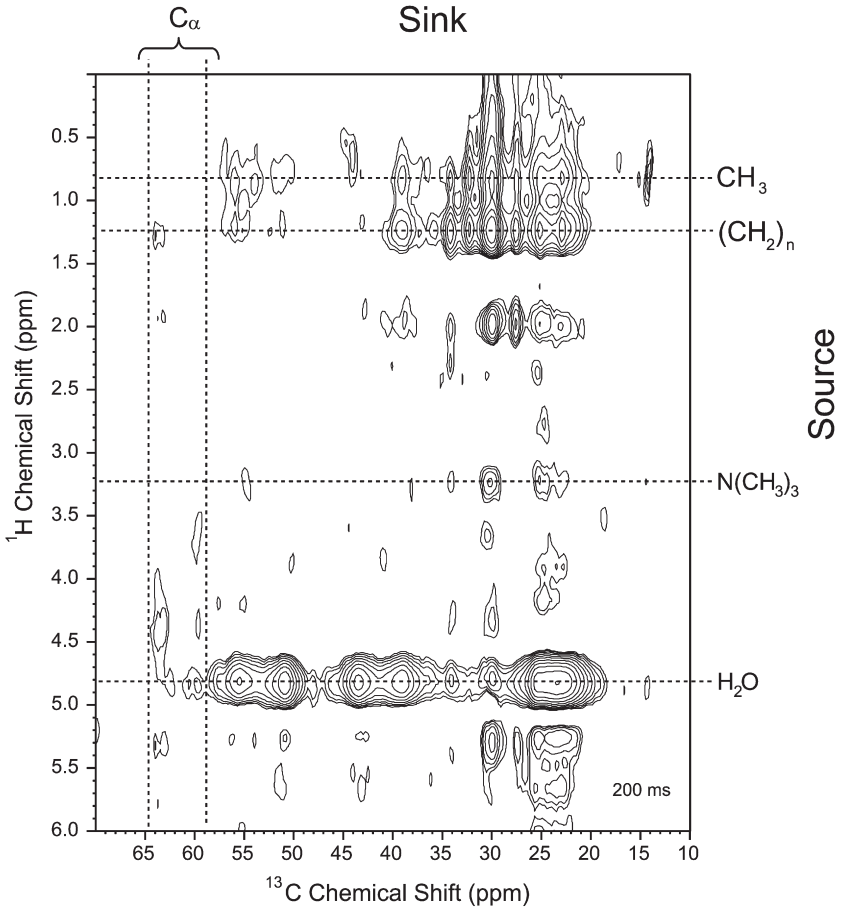

Fig. 5. 2-D ^{13}C-detected ^{1}H spin-diffusion spectrum of the membrane bound labeled L16 with a mixing time of 200 ms. A T_2 filter time of 2 ms, a relaxation delay of 3 s, and a spinning frequency of 7 kHz were used. The spectrum was recorded at 277 K. The peptide C_α region was determined from the CP/MAS data for the vesicle preparation shown in Fig. 3.

shows two signals at 172.5 ppm and 171.5 ppm. This splitting indicates slightly different signals for the ^{13}C carbonyl of the alternating Leu and Val residues in a unique overall conformation. Because the chemical shift is in the upfield region, the data indicate a β-sheet conformation. The resonance at 171.3 ppm observed for LV16–G8P9 also is indicative of predominantly β-sheet. This carbonyl line is asymmetric with a downfield extension at the base into the shift range for α-helix.

The ^{13}C 1D solid-state NMR spectra of the peptides in unoriented lipid vesicles show a similar trend in the ^{13}C-carbonyl chemical shifts as for the lyophilized samples (Fig. 3). The ^{13}C-carbonyl chemical shift of peptide L16 is at 175.2 ppm, indicating α-helix character for this peptide. The ^{13}C-carbonyl shift of peptides LV16 and LV16G8P9 is at 172.5 ppm in the upfield region, revealing β-sheet conformation. The relatively broad signals provide evidence for considerable structural disorder at the microscopic level in the peptide/lipid preparation. The heterogeneity appears most pronounced for LV16 and LV16–G8P9. This provides a view on the structure–function relation, since both peptides exhibit enhanced fusion-promoting activity. 1D ^1H spectra of the sample containing peptides bound to the lipids are depicted in Fig. 4. ^1H chemical shifts of the different lipid protons were assigned to H_2O (4.8 ppm), $N(CH_3)_3$ (3.4 ppm), NCH_2 (2.3 ppm), CH_2CO (2.5, 2.3 and 2.1 ppm), $(CH_2)_n$ (1.3–1.6 ppm) and CH_3-ω (1.0 ppm). Due to the low concentration of peptide, the peptide proton signals are not detected.

The lipid-initiated ^1H spin-diffusion technique provides a good approach to obtain topology information for membrane associated peptides in vesicles [5]. To determine the intercalation of the peptides with the membrane the considerable differences in the spin diffusion transfer rates between a rigid macromolecule and mobile lipids can be exploited [5]. A transmembrane peptide domain provides rigid segments close to the center of the liquid-crystalline bilayer, leading to rapid spin diffusion from the protons in the lipid tails to the peptide protons [5]. In contrast, for a peptide with only surface-bound segments, the ^1H magnetization must first diffuse through the lipid head groups before reaching the peptide [5]. In addition, since spin diffusion in lipids is slow as a result of motionally averaged ^1H–^1H dipolar couplings, a surface-bound peptide receives little ^1H magnetization from remote lipid chain protons [5]. The larger the distance between the peptide and the center of the bilayer, the slower is the spin diffusion [5,6]. The bilayers have an acyl chain length of about ~16 Å, a distance from the headgroup to the bilayer center of about ~22 Å, and a water layer thickness of about 19 Å. If a peptide is embedded in the bilayer according to the transmembrane model of Fig. 1A, it will

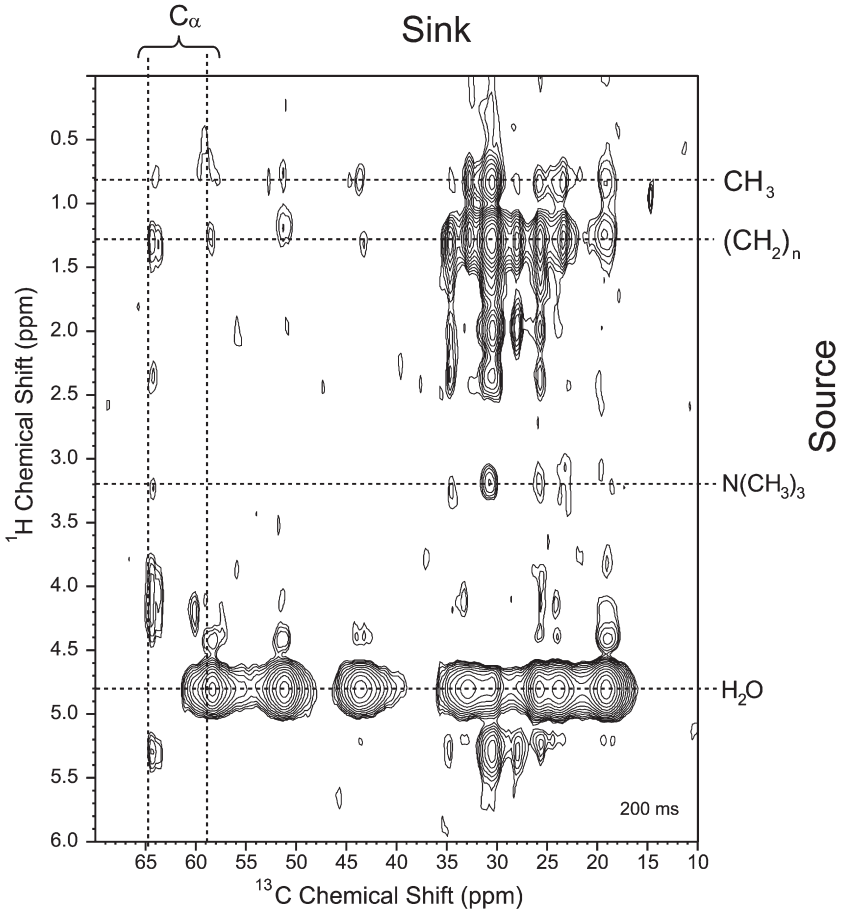

Fig. 6. 2-D ^{13}C-detected ^1H spin-diffusion spectrum with a mixing time of 200 ms of the membrane bound labeled LV16. A T_2 filter time of 2 ms, a relaxation delay of 3 s, and a spinning frequency of 7 kHz were used. The spectrum was recorded at 277 K. The peptide C_α sink region for the vesicle preparation is indicated with vertical dash lines.

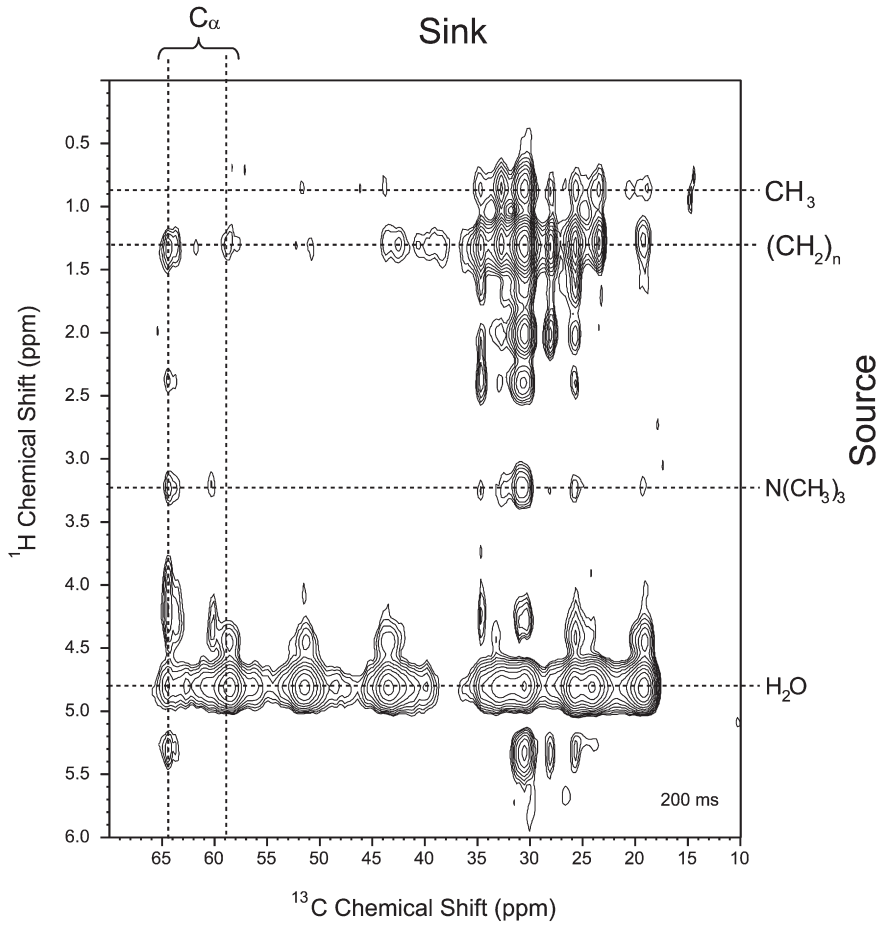

Fig. 7. 2-D ^{13}C-detected ^{1}H spin-diffusion spectrum of the membrane bound labeled LV16G8P9 using a mixing time of 200 ms. A T_2 filter time of 2 ms, a relaxation delay of 3 s, and a spinning frequency of 7 kHz were used. The spectrum was recorded at 277 K and the peptide C_α sink region for vesicles is indicated with vertical dash lines.

be in close proximity to the lipid chains and away from the H_2O. In contrast, when peptides are peripherally intercalated only part is buried in the bilayer while the remaining part is interacting with lipid headgroups. Such peptides are in close proximity to both the ^{1}H of the lipid chains and the H_2O protons.

Figs. 5, 6 and 7 show representative 2-D ^{13}C-detected ^{1}H spin-diffusion datasets for the membrane-bound peptides, L16, LV16 and LV16G8P9, respectively, obtained with a mixing time of 200 ms. Since a short contact time of 0.5 ms was used for CP for the peptide sample, the ^{13}C signals of the mobile lipids are mostly suppressed in the spectrum. Proper function of the T_2 filter was checked with τ_m=25 ms and only cross peaks with the mobile $(CH_2)_n$ segments of the lipids and H_2O correlation signals are observed (data not shown). Hence with 25 ms the ^{1}H signals of the more rigid peptide relax due to the strong ^{1}H–^{1}H dipolar couplings. The source and sink of spin diffusion are readily distinguished in the 2-D spectrum, since the ^{13}C chemical shifts of the heteronuclear correlation signals mark the sinks of spin diffusion while the ^{1}H chemical shifts indicate the sources of magnetization [5]. The strong peaks that are observed for H_2O protons are due to the correlations between water and lipid carbons, which are abundantly present in the sample.

To determine if a peptide is intercalated into the bilayer, the cross peak intensities for spin diffusion from the lipid chain methylene protons and H_2O protons to the C_α residues in the peptides were extracted from datasets with different spin diffusion mixing times. Table 1 shows the normalized build up intensities for the C_α residues for the fusogenic peptides LV16 and LV16G8P9, and for the non-fusogenic peptide, L16. Fig. 9 shows the build up curves for the Cα in the non-fusogenic peptide L16 and in the fusogenic peptides, LV16 and LV16G8P9. The solid circles represents the H_2O build up curve while open square depict the $(CH_2)_n$ build up curve. For

Table 1
Spin-diffusion build up for the C_α signal of LV16G8P9, LV16 and L16

τ_m ms	I.V16G8P9		I.V16		I.16	
	$(CH_2)_n$	H_2O	$(CH_2)_n$	H_2O	$(CH_2)_n$	H_2O
50	0.77±0.12	0.76±0.06	0.65±0.12	0.78±0.04	0.8±0.11	0.63±0.06
100	0.95±0.11	0.87±0.07	0.79±0.15	0.88±0.05	0.99±0.12	0.69±0.05
200	0.96±0.12	0.92±0.06	0.96±0.12	1±0.07	0.95±0.05	0.79±0.06
400	1±0.12	1±0.08	1±0.15	0.96±0.07	1±0.08	1±0.05

The magnetization sources are $(CH_2)_n$ and H_2O. Data were corrected for ^{1}H T_1 relaxation. Errors represent the noise level in the 2-D spectra.

all three peptides LV16G8P9, LV16 and L16 the normalized intensities for the C_α due to spin diffusion from the $(CH_2)_n$ show a fast build up on a time scale of ~100 ms, corresponding with a spin diffusion gap of ~2 Å, as reported for other peptides [5,6]. Also the normalized build up intensities for spin diffusion from the H_2O protons for the LV16G8P9 and LV16 peptides show the fast build up characteristic for a gap of ~2 Å [5,6]. In contrast, the signal build up by spin diffusion from the H_2O protons into peptide L16 is slow on a time scale of 400 ms [5,6]. The diffusion from H_2O into the LV16 at 100 ms diffusion time appears somewhat lower than for the LV16G8P9, which may indicate a more complicated system due to a partial transmembrane fraction, in line with recent FTIR results [20].

As the distance increases, magnetization is transferred along the lipid chains over an increasing distance, thus resulting in slower build-up curves(Fig. 9) [5]. Model studies have shown that diffusion over the entire lipid molecule, i.e. up to ~16 Å, corresponds with diffusion times in the range of 400 ms. Since the rate-limiting step of the proton spin diffusion is related to distances, the data indicate that the peptide L16 is closer to the protons of the lipid tails than to the H_2O [5]. Thus, the relatively slow build-up of the H_2O signal would be in line with a peptide fraction that is transmembrane and inserts all the way into the core of the hydrophobic bilayer (Fig. 1A). In contrast, the correlations from the samples loaded with fusogenic peptides LV16 and LV16G8P9 are probably from peptides at the membrane–water interface. The protons of the fusogenic peptides detected in these datasets are close to both the protons of the water molecules and the protons of the lipid chains (Fig. 1B).

According to the spin-diffusion data collected from vesicles loaded with peptides, the non-fusogenic L16 favors a transmembrane topology (cf. Fig. 1A) while the data for the fusogenic peptides are in line with peripheral intercalation (Fig. 1B). Transfer of magnetization from the lipid acyl chain to the peptide followed by redistribution within the peptide is in global agreement with the peripheral intercalation model for the fusogenic peptides, since it includes a membrane inserted part as well as a peptide domain interacting with the water layer. The data obtained are well in line with circular dichroism and IR measurements, which show that LV16G8P9 exists mainly in β-sheet and turns conformation in liposomes, which is also consistent with inducing curvature by peripheral association with the outer layer as depicted in Fig. 1B [20].

While the ^{15}N chemical shift of a peptide bond within a helical molecule exhibits values around 220 ppm when the helix long axis is oriented approximately parallel to the magnetic field direction, ^{15}N responses in the 60–80 ppm range are measured at perpendicular alignments [13]. If all directions are present in a sample, such as in a solid powder, then all of the different degrees of shielding, and their resulting chemical shifts, are observed [13]. Assuming that a peptide binds and inserts into lipid bilayers with a specific orientation, then all the peptides in the aligned bilayer samples will be lined up the same way, and a single narrow peak will be observed in the ^{15}N spectrum [13].

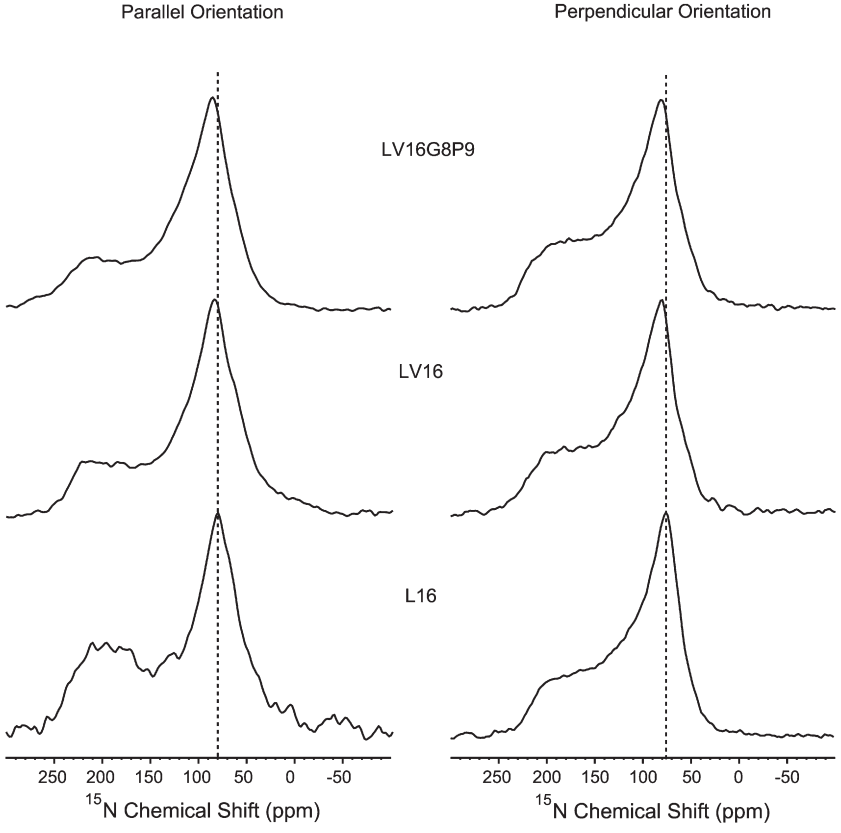

Fig. 8. Proton-decoupled ^{15}N spectra of L16, LV16 and LV16G8P9 reconstituted into oriented biomimetic membranes where the bilayer normal parallel or perpendicular to the magnetic field direction. The data reveal quenching of rotational diffusion for all peptides, most probably due to aggregation, with a slight indication for transmembrane orientation for L16.

Since σ_{33} of the ^{15}N shift tensor is oriented close to the N−H bond and helix axis, a peptide α-helix with its axis parallel to the magnetic field will have a chemical shift near $\sigma_{||}$, and a peptide α-helix with its axis perpendicular to the magnetic field will have a chemical shift near $\sigma_{\perp}$ [13].

The ^{15}N shifts for the solid-state NMR data collected from macroscopically aligned bilayer samples loaded with uniformly labeled peptides with the normal parallel to B_0 cover the full anisotropy range between 60 and 230 ppm with little averaging of the shift anisotropy (Fig. 8). These NMR measurements have been performed at 310 K, i.e. well above the gel-to-liquid crystalline phase transition of the lipids used. The proton decoupled ^{15}N spectrum of the non-fusogenic peptides L16 in bilayers shows a powder pattern with a broad peak at 200 ppm that is intriguing, since the shift corresponds with the $\sigma_{||}$ for the signal of a monomeric transmembrane motif. When the samples are rotated by 90°, with the bilayer normal perpendicular to the magnetic field, the ^{15}N NMR spectra of the peptides show a powder pattern indicating a random distribution of orientations in the sample, as expected for peptide aggregates (Fig. 8, right panels).

The spectra shown in Fig. 8 were collected from oriented bilayer samples loaded with peptides, which is different from the vesicle preparations used in the spin diffusion experiments. It is remarkable that for the L16 peptide a marginally enhanced $\sigma_{||}$ signal is observed, at the shift value expected for transmembrane peptides. This correlates with the observation of a transmembrane fraction in the spin-diffusion dataset for the vesicles loaded with L16 peptide.

4. Conclusions

In conclusion, with relatively straightforward solid-state NMR experiments on systems that mimic closely the natural system, useful information about the relation between structure and molecular mechanisms of importance for the function is obtained for a set of *de novo* designed fusogenic peptides in interaction with their lipid environment. Peptides incorporated in a membrane that can catalyze fusion processes exhibit peripheral intercalation, while for the non-fusogenic L16 evidence for an embedded fraction with transmembrane characteristics is obtained. The data contribute to converging evidence that peripheral intercalation can trigger the cooperative phenomena that lead to outer leaflet mixing and fusion pore formation, probably by inducing phase separation across the lipid bilayer [4].

For oriented samples, the spectral line shapes are sensitive indicators of the rate of rotational diffusion. While rapidly rotating transmembrane peptides exhibit spectral averaging and well-defined resonances, broad spectral line shapes characterize larger immobilized complexes. The ^{15}N NMR data provide evidence for peptide aggregation in lipid bilayers with orientation distributions. The intercalated part actually represents only a fraction of the total sample, and this would imply that a major fraction is not detected in the 2D HETCOR experiments. In addition, the data shown in Fig. 9 cannot be analyzed with a single buildup curve, which suggests also a heterogeneous distribution of peptides and is in line with the recently published study of a fusogenic peptide, pardaxin [21].

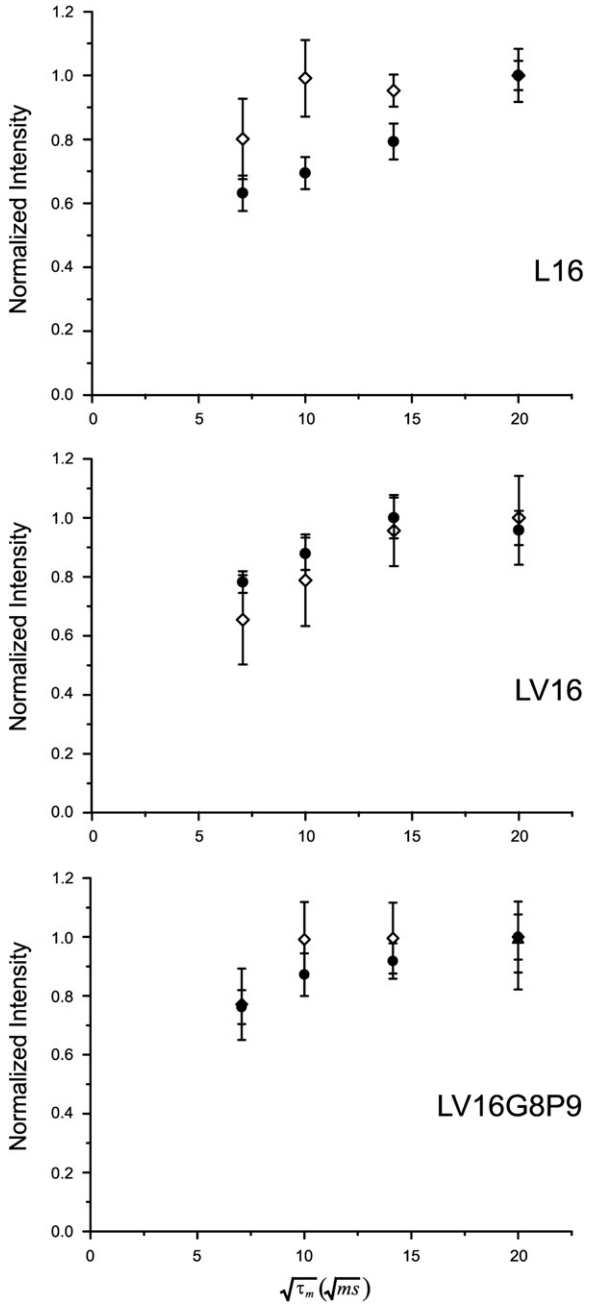

Fig. 9. Spin-diffusion build up versus mixing time for the L16, LV16 and LV16G8P9 C_α signals. The magnetization sources are H_2O (solid circle) and $(CH_2)_n$ (open square). The data were corrected for ^1H T_1 relaxation. The error bars represent the estimated noise levels of the signals in the 2-D spectra.

The biomimetic lipid environment setup can be used as a screening tool for many different peptides, proteins and other organic and bio-molecules. Future studies will show whether the fusogenicity of peptides correlates with their detailed secondary structure and conformational flexibility in the membrane environment.

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