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Membrane interactions and dynamics of a 21-mer cytotoxic peptide: A solid-state NMR study

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ABSTRACT

We have investigated the membrane interactions and dynamics of a 21-mer cytotoxic model peptide that acts as an ion channel by solid-state NMR spectroscopy. To shed light on its mechanism of membrane perturbation, ^{31}P and ^{2}H NMR experiments were performed on 21-mer peptide-containing bicelles. ^{31}P NMR results indicate that the 21-mer peptide stabilizes the bicelle structure and orientation in the magnetic field and perturbs the lipid polar head group conformation. On the other hand, ^{2}H NMR spectra reveal that the 21-mer peptide orders the lipid acyl chains upon binding. ^{15}N NMR experiments performed in DMPC bilayers stacked between glass plates also reveal that the 21-mer peptide remains at the bilayer surface. ^{15}N NMR experiments in perpendicular DMPC bicelles indicate that the 21-mer peptide does not show a circular orientational distribution in the bicelle planar region. Finally, ^{13}C NMR experiments were used to study the 21-mer peptide dynamics in DMPC multilamellar vesicles. By analyzing the ^{13}CO spinning sidebands, the results show that the 21-mer peptide is immobilized upon membrane binding. In light of these results, we propose a model of membrane interaction for the 21-mer peptide where it lies at the bilayer surface and perturbs the lipid head group conformation.

1. Introduction

The study of membrane-active peptides has become the focus of many research groups since they represent potential antimicrobial agents acting as substitutes to ineffective antibiotics for which bacteria have developed resistance mechanisms [1–5]. Part of the defense system of many living organisms, natural membrane-active peptides share common properties such as their small size, and their cationic and amphipathic characters, the latter being essential to their interactions with amphipathic lipid bilayers [6–9]. When interacting with membranes, antimicrobial peptides can adopt different secondary structures, such as α-helix, β-sheet, and extended conformations [10,11], and their membrane interactions can be modulated by varying peptide structural parameters such as helicity, charge, hydrophobicity, and amphipathicity [7,12].

Most of antimicrobial peptides possess a broad spectrum of activity, i.e., acting on viruses, bacteria, and fungi [6,9,13]. However, peptides such as melittin, magainin, lactoferricin B, cecropin B, and citropin 1.1 show cytotoxic properties against mammalian cells by lysing erythrocytes and killing cancer cells [14–21]. Based on extensive studies of natural and synthetic membrane-active peptides, general modes of action are reported in the literature, namely, the carpet-like, the barrel-stave, and the toroidal models [22–25]. Because these mechanisms of membrane perturbation are not fully understood and still under debate, the focus is then turned on the studies of antimicrobial peptides in interaction with model and biological membranes to get more information on the interactions underlying these mechanisms [1,24,26,27]. A better understanding of how membrane-active peptides interact with bilayers opens the way to the design of novel membrane-active agents that possess the desired specific biological action, being cytotoxic and/or antimicrobial [28–30].

Inspired by the great potentials of membrane-active peptides as novel therapeutic agents, many research groups have focused on the design of synthetic ion channels with the aim to better understand both structural features and the types of interactions involved in the channel activity [17,31–33]. In this way, we have designed and synthesized a synthetic helical amphipathic peptide composed of 21 amino acids [34–36]. The 21-mer peptide is a trimer of a repeating unit of five leucine residues and two synthetic 21-crown-7-phenylalanines judiciously positioned so that the hydrophilic crown ethers align on one side of the hydrophobic helix under a helical conformation, giving rise to an amphipathic peptide structure (Fig. 1).

Previous studies revealed that the 21-mer peptide acts as an artificial ion channel when incorporated into lipid bilayers [35,36]. To shed light on its membrane properties, we have studied the interactions between the 21-mer peptide and model membranes by solid-state nuclear magnetic resonance (NMR) spectroscopy. Solid-state NMR is a well-suited technique to study both the lipid bilayers and peptides by taking advantage of the orientational dependence of

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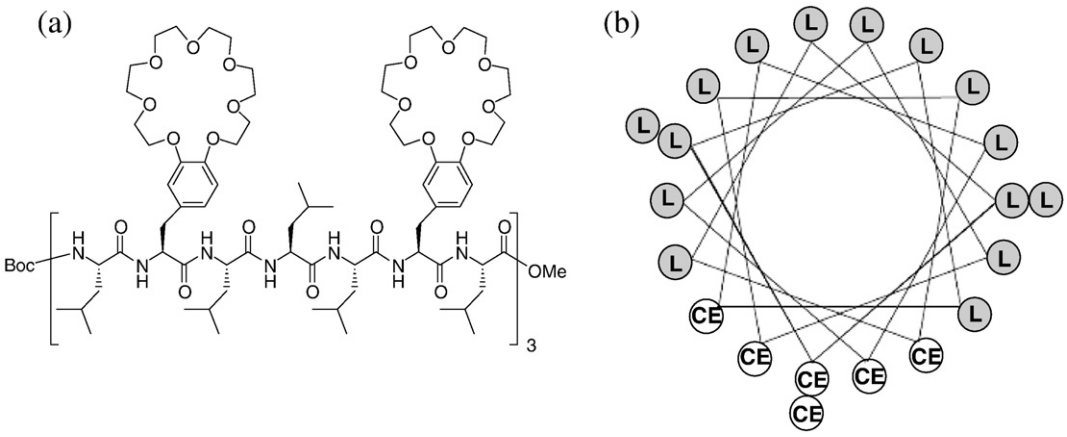

Fig. 1. (a) Diprotected 21-mer peptide structure used in this study. (b) Edmundson wheel projection showing the amphipathic character of the 21-mer peptide under a helical conformation.

anisotropic nuclear spin interactions in the static mode [37–39]. Alternatively, magic-angle spinning (MAS) is commonly used to obtain high-resolution NMR spectra that allow the study of peptide structure and dynamics [40–42]. More specifically, we report the membrane interactions of the 21-mer peptide with oriented model membranes. Static ^{31}P NMR spectroscopy has been used to determine the magnetic orientation of dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) bicelles, and the lipid polar head group conformation upon the 21-mer peptide binding, whereas static ^{2}H NMR experiments have been used to investigate DMPC and DPPC lipid acyl chain orientational order. Subsequently, ^{15}N NMR experiments have been performed on both DMPC bicelles and bilayers stacked between glass plates to determine both the peptide rotational diffusion and membrane orientation. Finally, ^{13}C NMR experiments have allowed the study of the 21-mer peptide dynamics when incorporated to multilamellar DMPC vesicles by the analysis of spinning sideband intensity. The results are in agreement with a model of membrane interaction where the helicoidal peptide binds parallel to the bilayer surface, does not show a circular orientational distribution around the bilayer normal, and perturbs the lipid polar head group conformation.

2. Materials and methods

2.1. Materials

DMPC with protonated and deuterated (d_{54}) acyl chains, dihexanoylphosphatidylcholine (DHPC), and DPPC were purchased from Avanti Polar Lipids (Alabaster, AL) and used without purification. Oxime resin was prepared by a standard procedure using polystyrene beads (100–200 mesh 1% DVB, Advanced ChemTech, Louisville, KY) [43]. Resins with substitution levels around 0.5 mmol/g of oxime group were used. Boc-protected amino acids were purchased from Advanced ChemTech (Louisville, KY). The ^{15}N and ^{13}CO leucine residues were purchased from Cambridge Isotope Laboratories (Andover, MA). All solvents were Reagent, Spectro, or HPLC grade quality purchased commercially and used without any further purification except for DMF (degassed with N_2), dichloromethane (distilled), and diethyl ether (distilled from sodium and benzophenone). Water used throughout the studies was distilled and deionized using a Barnstead NANOpurII system (Boston, MA) with four purification columns. All other reagents were purchased from Sigma Aldrich Co. (Milwaukee, WI).

2.2. Peptide synthesis

The unlabeled and ^{15}N, ^{13}C-labeled diprotected 21-mer peptides (Fig. 1a) were synthesized and purified according to published procedures [34]. The N-terminal region of the 21-mer peptide is Boc protected while the C-terminal region is acetylated (Fig. 1a). Previous membrane activity studies have been done on various 21-mer peptides with different N- and C-terminal groups, and the results indicate that the N-Boc and C-acetylated 21-mer peptide show the greatest ion channel activity (Biron et al., unpublished results).

2.3. Sample preparation

2.3.1. Bicelles

Bicelle samples were prepared by mixing 20 mg of long-chain phospholipids (DMPC or DPPC) with DHPC to achieve the desired long-chain:short-chain phospholipid ratios ($q = 3.5$ for DMPC, $q = 3.0$ for DPPC) before sample hydration [44]. For ^{2}H NMR experiments, 5 mg of deuterated DMPC-d_{54} and DPPC-d_{62} was used to replace 5 mg of undeuterated lipids. A total of 80 μL of deuterium-depleted water was used, giving a total proportion of 20% (w/w) lipids in water. This corresponds to 155 water molecules per lipid molecule and such system can be qualified as fully hydrated. The pH was ~6.5 in all samples. The 21-mer peptide was added after the bicelle formation and hydration in a lipid/peptide molar ratio of 60:1. The bicelle samples then underwent at least three freeze (liquid N_2)/thaw (37 °C for DMPC; 50 °C for DPPC)/vortex shaking cycles, and were stored at −20 °C until analysis. Three additional freeze/thaw/vortex cycles were performed before the acquisition of NMR spectra.

2.3.2. Oriented bilayers stacked between glass plates

The DMPC bilayers were prepared by dissolving 30 mg of phospholipids in 120 μL of chloroform, and the solution was deposited onto 18 thin cover glasses. The glass plates were allowed to dry in air for 24 h, and then stacked and hydrated with deionized water in a closed chamber for at least 24 h at 70 °C. Subsequently, the plates were wrapped in Parafilm before use. For the preparation of the peptide-containing bilayers, the dry 21-mer peptide was co-dissolved with dry lipids in chloroform at a lipid/peptide molar ratio of 60:1. Since the lipids found in natural membranes are in the fluid phase, the NMR spectra of mechanically oriented bilayers have been obtained at a temperature above the lipid phase transition, i.e., at 37 °C for DMPC bilayers.

2.3.3. Multilamellar vesicles

Multilamellar vesicles were prepared by co-dissolving 30 mg of DMPC and 10 mg of doubly labeled [^{15}N]Leu$_{15}$-[^{13}CO]Leu$_{18}$-21-mer peptide in chloroform to ensure homogeneization of the lipid/peptide mixture. The solvent was removed under nitrogen gas, followed by storage under vacuum overnight to remove all traces of organic solvent. The dry sample was hydrated with 46 μL of deionized water

with 40% (w/w) lipids in water. The vesicle samples then underwent at least three freeze (liquid N_2)/thaw (37 °C)/vortex shaking cycles, and were stored at −20 °C until analysis. Three additional freeze/thaw/vortex cycles were performed before the acquisition of ^{13}C NMR spectra.

2.4. NMR experiments

2.4.1. Static ^{31}P and ^{2}H NMR experiments

The static ^{31}P and ^{2}H NMR spectra of the 21-mer peptide incorporated in bicelles and in mechanically oriented DMPC bilayers were acquired with a Bruker Avance 300 MHz spectrometer (Bruker Biospin Ltd., Milton, Ontario, Canada). The bicelle samples were placed into a 5-mm coil of a homebuilt probe. The thin glass plates were inserted into a flat coil of a homebuilt solid-state NMR probehead with the glass plate normal oriented parallel to the magnetic field direction. The ^{31}P NMR spectra of bicelles were obtained at 121.5 MHz using a phase-cycled Hahn echo pulse sequence [45] with TPPM proton decoupling [46]. The ^{31}P NMR spectra of oriented DMPC bilayers were acquired with TPPM proton decoupling. Using 4096 data points, typically 1200 scans were acquired with a pulse length of 4 μs, an interpulse delay of 30 μs, and a recycle delay of 4 s. A line broadening of 50 Hz was applied to all static ^{31}P spectra. The chemical shifts were referenced relative to external H_3PO_4 85% (0 ppm). The ^{2}H NMR experiments were carried out at 46.1 MHz using a quadrupolar echo sequence [47]. The 90° pulse length was 5 μs and the interpulse delay was 60 μs. Using 2048 data points, typically 3200 scans were acquired, and the recycle time was set at 500 ms. A line broadening of 100 Hz was applied to all spectra. A 1800-s equilibration delay was allowed between each temperature in the ^{31}P and ^{2}H experiments.

2.4.2. Static ^{15}N NMR experiments

The static ^{15}N NMR spectra of the 21-mer peptide incorporated in bicelles and in glass plates were acquired with a Bruker Avance 300 MHz spectrometer (Bruker Biospin Ltd., Milton, Ontario, Canada). The bicelle sample was placed into a 7-mm coil of a Bruker MAS probe, whereas the glass plate sample was inserted into a flat coil of a homebuilt solid-state NMR probehead with the glass plate normal oriented parallel to the magnetic field direction. The ^{15}N NMR spectra were obtained at 30.4 MHz using a cross-polarization (CP) pulse sequence with TPPM proton decoupling [46]. Using 2048 data points and a recycle delay of 4 s, between 100,000 and 200,000 scans were acquired with a $^{1}H^{15}N$ CP contact time of 1.5 ms and 3.0 ms for the 21-mer peptide in bicelles and in oriented DMPC bilayers. The spectral width was 30 kHz and a line broadening of 300 Hz was applied to all ^{15}N oriented spectra. The chemical shifts were referenced relative to external $^{15}NH_4Cl$ (41.5 ppm) corresponding approximately to 0 ppm for liquid NH_3.

2.4.3. MAS ^{13}C NMR experiments

^{13}C NMR spectra were acquired with a Bruker Avance 400 MHz spectrometer (Bruker Biospin Ltd., Milton, Ontario, Canada). The samples were placed into a 4-mm NMR tube inserted into a MAS probe. The ^{13}C NMR spectra were obtained at 100.6 MHz using a ramped cross-polarization pulse sequence with TPPM proton decoupling [46]. The ramp used 100 linearly increasing rf amplitudes and was applied on the ^{1}H channel. A short CP contact time of 0.5 ms was used to minimize lipid background signals. The spinning speeds were set at 5000 Hz, and to 2500 Hz to generate spinning sidebands. Using 2048 data points, between 5000 and 10,000 scans were acquired with a recycle delay of 4 s. The spectral width was 50 kHz and a line broadening of 150 Hz was applied to all ^{13}C NMR spectra. The acquisition temperature for hydrated samples was set at 37 °C. The chemical shifts were referenced relative to tetramethylsilane (TMS) at 0 ppm.

3. Results and discussion

3.1. Peptide design

The rational design of the 21-mer peptide was to use a peptidic framework sufficiently hydrophobic to be incorporated in lipid bilayers and long enough to span the bilayer without hydrophobic mismatch. Leucine and phenylalanine residues have been chosen as constituents of the peptide backbone because of their relatively high hydrophobic moment and propensity to favor a α-helical structure. The helical conformation of the 21-mer peptide has been confirmed by circular dichroism spectropolarimetry [35]. Also, phenylalanine residues have been modified to add 21-crown-7 macrocyclic ethers on the benzene ring, as illustrated in Fig. 1a. The 21-crown-7 ethers were incorporated judiciously at positions 2, 6, 9, 13, 16, and 20 in the amino acid sequence to lead to their alignment under a α-helical conformation. In addition, 21-crown-7 ligands bind alkali metal ions rather poorly, which is a prerequisite for an ion channel activity, and their binding ability can be engineered by their size modulation [48,49]. The spatial segregation of polar 21-crown-7 ethers on one side of the helix and the hydrophobic leucine side chains on the other side confers to the 21-mer peptide an amphipathic character essential to its interaction with amphipathic membranes (Fig. 1b). Assuming that the 21-mer adopts a perfect α-helical conformation with 1.5 residues per turn and a helical step of 3.4 Å, its length is 31.5 Å.

The membrane transport activity of the 21-mer peptide has previously been studied by ^{23}Na NMR, fluorescence spectroscopy, pH-stat, and monomolecular membrane conductivity. Based on the differentiation of Na^+ inside and outside lipid vesicles by the use of Dy^{3+} as shift reagent, ^{23}Na NMR studies have revealed that the 21-mer peptide incorporated in PC/PG vesicles allows an exchange of Na^+ across the bilayer similar to the parent 14-mer peptide investigated in our research group [34]. However, fluorescence studies revealed that the 21-mer peptide does not release calcein from PC/PG vesicles in a similar way that the 14-mer peptide does [50]. These results confirmed that the activity observed in the ^{23}Na NMR assay reflects the transport ability of the 21-mer artificial channel. To further characterize the transport ability of the 21-mer peptide, both pH-stat and monomolecular membrane conductivity experiments have been performed in PC/PA/cholesterol and DPPC bilayers [35,36]. Both experiments revealed that the 21-mer peptide exhibits transport activity for Cs^+, Li^+, Na^+, K^+, and Rb^+ ions and shows typical discrete conductance steps of 3.5 ± 0.2 pA with open state lifetimes around 1 s. Preliminary antimicrobial and cytotoxicity studies have also been done on *Escherichia coli* and *Pseudomonas aeruginosa* Gram-negative bacteria, on *Staphylococcus aureus* and *Streptococcus pneumoniae* Gram-positive bacteria, on breast cancer cells (MDA), and on mouse leukemia cells (P388) [34]. No antimicrobial activity was found for the 21-mer peptide, whereas it shows some activity against cancer cells. In addition, the 21-mer peptide activity assay on human red blood cells revealed that the 21-mer peptide releases hemoglobin from erythrocytes at a minimal concentration of 8 μM, which is twice the minimal concentration observed for the 14-mer peptide to cause the same biological effect (Biron et al., unpublished results). In light of the membrane activity of the 21-mer peptide in both model membranes and cells, ^{31}P and ^{2}H NMR experiments on unoriented zwitterionic and anionic vesicles were performed [51]. The results showed that the 21-mer peptide affects the lipid polar head group conformation, whereas it does not affect the lipid acyl chain order.

3.2. Solid-state NMR spectroscopy

3.2.1. Phosphorus-31 NMR

Since the phospholipid head group contains a phosphorus-31 atom with 100% natural isotopic abundance, ^{31}P NMR is a powerful technique to monitor dynamics and/or orientational changes at the

polar region of lipid bilayers [52–54]. In the present study, we have used ^{31}P NMR spectroscopy to investigate the quality of magnetic orientation of bicelles composed of different long-chain lipids, namely, DMPC and DPPC, upon the addition of the 21-mer peptide, as well as conformational changes occurring at the bicelle head group region. These experiments are of great interest since bicelles are well-hydrated bilayers and represent a good way to orient peptides in the magnetic field prior to rotational diffusion and membrane topology studies [55–58]. The choice of long-chain lipids with different acyl chain lengths was aimed to determine the effect of the bilayer hydrophobic thickness on the membrane interaction of the 21-mer peptide. The DMPC and DPPC bicelle thicknesses are evaluated to be 23.0 and 26.0 Å, respectively [44].

More specifically, static ^{31}P NMR spectra provide information on bicelle orientation and on the dynamics and/or orientation of the lipid polar head group by analyzing changes in the chemical shift (δ, ppm), in the linewidth, and in the spectral line shape. Picard et al. proposed a way to quantitatively assess the level of bicelle orientation by evaluating a relative order parameter of the form [59,60]:

$$S_1 = S_{\text{dist}} \times S_{\text{tilt}} \tag{1}$$

where

$$S_{\text{dist}} = \frac{(M_1 - \delta_{\text{iso}})}{\delta} \tag{2}$$

The S_{dist} term is defined as a distribution order parameter. M_1 is the first spectral moment of ^{31}P NMR spectra representing the weighted-average DxPC spectral frequency, and δ_{iso} and δ are the DxPC isotropic chemical shift and the chemical shift anisotropy, DxPC being either DMPC or DPPC lipids. The S_1 order parameter for perpendicular bicelles is obtained by multiplying the S_{dist} order parameter by a S_{tilt} order parameter value of −0.5. Any deviation of the S_1 order parameter from the −0.5 value indicates that the bicelle orientation deviates from the perfect perpendicular orientation. Picard et al. also monitored changes in the lipid head group dynamics and/or orientation by measuring a relative order parameter of the form:

$$S_2 = \frac{\delta}{\delta_{\text{ref}}} \tag{3}$$

in which δ and δ_{ref} are the DxPC chemical shift anisotropies of the peptide-containing and pure bicelles [60]. The S_2 order parameter is equal to 1 for peptide-containing bicelles in which the dynamics is the same as in the reference system, and to 0 for a totally isotropic system. A S_2 value greater than 1 may also be obtained for a less dynamic lipid system compared to the pure lipid system. As previously reported, bicelles are represented as discoid bilayers or perforated lamellae that spontaneously align with the bilayer normal perpendicular to the magnetic field direction [61–64]. In the temperature range of bicelle orientation, two well-resolved resonances are observed in ^{31}P NMR spectra [65,66]. The downfield and upfield resonances are mainly attributed to short-chain lipids (DHPC) located on the bicelle torus and to long-chain lipids (DMPC or DPPC) located in the planar region [62,63,66,67], respectively. In a general manner, downfield shifts of the resonances upon peptide addition could originate from changes in the phosphorus nucleus environment by the interaction with peptide deshielding moieties while upfield shifts could originate from the interaction with peptide shielding moieties. These changes could also be related to changes in head group orientation and/or dynamics.

^{31}P static NMR spectra and related spectral parameters of pure and peptide-containing bicelles are displayed in Fig. 2 and Table 1. Oriented bicellar systems are known to exist in a specific temperature range over the long-chain lipid phase transition temperature T_m, and their structure and orientation are function of the bicelle size and lipid composition [65,68,69]. ^{31}P NMR spectra of DMPC and DPPC bicelles have been recorded at different temperatures above T_m to probe the 21-mer peptide effect in the temperature range in which bicelles conserve their structure and perpendicular orientation. ^{31}P NMR spectra of DMPC bicelles have been recorded at 30, 35, 37, and 40 °C, whereas ^{31}P spectra of DPPC bicelles have been recorded at 40, 42, 45, and 47 °C.

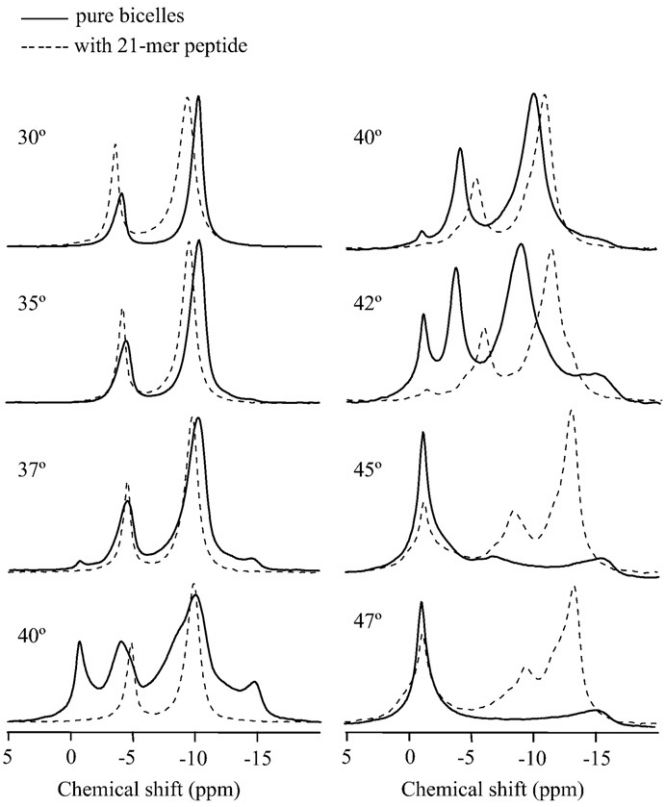

Fig. 2. ^{31}P static NMR spectra of (left) DMPC and (right) DPPC bicelles at different temperatures and in the absence and presence of the 21-mer peptide at a lipid/peptide molar ratio of 60:1.

As shown in Fig. 2, the ^{31}P NMR spectra of perpendicular bicelles are characterized by the presence of two resonances. In the case of the DMPC bicelles, the DHPC and DMPC resonances are observed at chemical shifts of −4.6 and −10.4 ppm, and the DMPC bicelles are aligned in the magnetic field in the temperature range between 30 and 37 °C. At 40 °C, the ^{31}P NMR spectrum of DMPC bicelles is no longer representative of perpendicular bicelles. For DPPC bicelles, DHPC and DPPC resonances appear at −4.1 and −10.1 ppm, and the bicelles are only aligned at a temperature close to the phase transition temperature of the DPPC lipids, namely, 40 °C. Above that temperature, the bicelle structure is gradually lost leading to the appearance of an isotropic resonance at −1.0 ppm. The addition of the 21-mer peptide to DMPC and DPPC bicelles seems to stabilize the structure

Table 1
^{31}P NMR spectral parameters of bicelles in the absence and presence of the 21-mer peptide.

System	DHPC (δ, ppm)	DxPC (δ, ppm)	S_1	S_2
DMPC bicelles	−4.6	−10.4	−0.34	0.92
+21-mer	−4.3	−9.6	−0.33	
DPPC bicelles	−4.1	−10.1	−0.34	1.1
+21-mer	−5.5	−11.0	−0.35	

DxPC represents DMPC or DPPC lipids. Data are reported at 35 °C for DMPC bicelles and at 40 °C for DPPC bicelles. The lipid/peptide molar ratio is 60:1.

and prevents the bicelles to break into micelles or multilamellar vesicles. This is reflected in Fig. 2 where two resonances are still present in the ^{31}P NMR spectra of DMPC (left) at 40 °C and of DPPC (right) bicelles at 42, 45, and 47 °C upon peptide addition, compared to distorted spectral line shape of the pure lipid systems at these temperatures. In addition to the bicelle structure stabilization, the 21-mer peptide does not affect significantly the bicelle orientation at temperatures of bicelle existence, with S_1 value differences of 0.01 for both DMPC and DPPC bicelles at 35 and 40 °C, respectively. Interestingly, Triba et al. studied the correlation between the propensity of short-chain lipids to diffuse into the bilayer domain composed mainly of long-chain lipids and the alignment property of bicelles by ^{31}P NMR [69]. More specifically, by studying spectral line shapes of DHPC/DLPC and DHPC/DPPC bicelles as a function of temperature for a q ratio of 3.0, they observed that the temperature range in which bicelles align in the magnetic field is related to the propensity of DHPC to diffuse into the bilayer domain, and that miscibility is influenced by the mismatch between lipid chain lengths, the T_m of long-chain lipids, and the affinity of short-chain lipids for the torus. According to the conclusions drawn from Triba's studies, we may suppose that the increase in the temperature range of DMPC and DPPC bicelle orientation upon the 21-mer peptide addition is explained by the fact that the 21-mer peptide increases the miscibility of DHPC lipids into the DMPC and DPPC bilayer region.

In the temperature range of bicelle orientation, namely, 30–37 °C for DMPC bicelles and 40 °C for DPPC bicelles, the ^{31}P NMR spectra show that the DHPC and DMPC resonances in DMPC bicelles (left) are downfield shifted, with the exception of the DHPC resonance, which remains unchanged at 37 °C, whereas the DHPC and DPPC resonances in DPPC bicelles (right) are upfield shifted upon the 21-mer peptide addition. As shown in Table 1, downfied shifts of 0.3 and 0.8 ppm are reported for DHPC and DMPC in DMPC bicelles at 35 °C, whereas upfield shifts of 1.4 and 0.9 ppm are reported for DHPC and DPPC in DPPC bicelles at 40 °C.

The downfield and upfield shifts observed for DMPC and DPPC bicelles could originate from the interaction of phospholipid head groups with deshielding or shielding moieties in the 21-mer peptide or from changes in head group dynamics and/or orientation [51,70]. In the former case, the lipid isotropic chemical shift, which is expected to change, can be verified by MAS, otherwise the δ_{iso} is unchanged. ^{31}P MAS experiments performed in both bicelle systems indicated that the δ_{iso} and resonance linewidth remain unchanged upon the 21-mer peptide addition (data not shown). The downfield and upfield shifts observed for DMPC and DPPC bicelles, respectively, could therefore be attributed to disorder or order or to orientational changes in the phospholipid head groups in interaction with the 21-mer peptide. Similar conclusions were drawn by Marcotte et al. who studied the neuropeptide Menk bound to zwitterionic and anionic bicelles [70].

Table 2
Quadrupolar splittings and S_{CD} order parameters of DMPC-d_{54} and DPPC-d_{62} bicelles at the plateau and methyl regions in the absence and presence of the 21-mer peptide.

System	$\Delta\nu_{Q\ (P)}$ (kHz)	$\Delta\nu_{Q\ (M)}$ (kHz)	$S_{CD\ (P)}$	$S_{CD\ (M)}$
DMPC bicelles	20.0	2.8	0.16	0.02
+21-mer	21.0	2.6	0.17	0.02
DPPC bicelles	19.0	2.4	0.15	0.02
+21-mer	21.6	2.8	0.17	0.02

$\Delta\nu_Q$ and S_{CD} data are reported for DMPC and DPPC bicelles at 35 °C and 40 °C, respectively. The lipid/peptide molar ratio is 60:1.

Therefore, it appears that the 21-mer peptide stabilizes the bicelle structure and induces disorder in the DMPC bicelles while it induces order in the DPPC bicelles. These differences could originate from the physical properties of bicelles with various long-chain lipids.

3.2.2. Deuterium NMR

We have investigated the effect of the 21-mer peptide on the bicelle hydrophobic core by measuring the quadrupolar splitting $\Delta\nu_Q$ upon the peptide addition. The lipid acyl chain order is related to an order parameter S_{CD} that takes the following form:

$$\Delta\nu_Q = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)(3\cos^2\theta - 1)S_{CD} \quad (4)$$

where e^2qQ/h is the quadrupole coupling constant for C–D bonds (~167 kHz) and θ is the angle between the bilayer normal and the magnetic field B_0 [53]. An increase in the quadrupolar splitting $\Delta\nu_Q$ for a C–D bond in a lipid system with axial symmetry is related to order in the deuterated chains while a decrease in the quadrupolar splitting is related to disorder [53,71,72]. Quadrupolar splittings $\Delta\nu_{Q\,(P)}$ and $\Delta\nu_{Q\,(M)}$ are calculated for deuterons at the beginning and at the end of the lipid acyl chains, namely, the plateau and terminal methyl regions.

The ^{2}H NMR spectra and related spectral parameters obtained for pure and peptide-containing DMPC and DPPC bicelles at 35 and 40 °C are displayed in Fig. 3 and Table 2. ^{2}H NMR spectra exhibit typical line shapes of perpendicular-aligned bicelles with quadrupolar splittings for the plateau region ($\Delta\nu_{Q(P)}$) of 20.0 kHz and 19.0 kHz, which correspond to $S_{CD\ (P)}$ order parameter values of 0.16 and 0.15 for DMPC and DPPC bicelles, respectively. The quadrupolar splittings for the methyl groups at the end of the lipid acyl chains ($\Delta\nu_{Q(M)}$) are 2.8 and 2.4 kHz, which correspond to $S_{CD(M)}$ order parameter values of 0.02 for both the DMPC and DPPC bicelles.

Upon the 21-mer peptide addition at a lipid/peptide molar ratio of 60:1, the $\Delta\nu_{Q(P)}$ and $S_{CD(P)}$ values increase by 1.0 kHz and 0.01 for DMPC bicelles, whereas the effect is even more pronounced in DPPC bicelles with $\Delta\nu_{Q(P)}$ and $S_{CD(P)}$ increases of 2.6 kHz and 0.02. In both

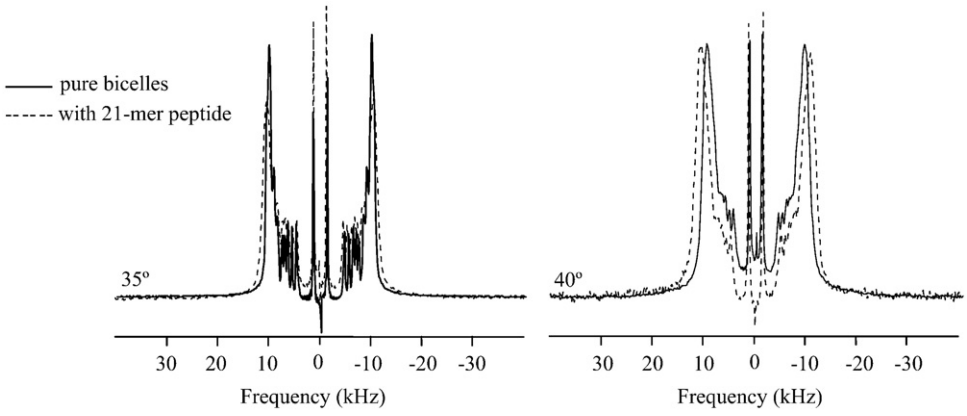

Fig. 3. ^{2}H NMR spectra of (left) DMPC and (right) DPPC bicelles at 35 °C and 40 °C in the absence and presence of the 21-mer peptide at a lipid/peptide molar ratio of 60:1.

bicelle systems however, $\Delta\nu_{Q(M)}$ and $S_{CD(M)}$ do not change significantly, with a slight decrease of 0.2 kHz in DMPC bicelles and a slight increase of 0.4 kHz in DPPC bicelles. These results suggest that the model peptide interacts preferentially at the bilayer surface, ordering the plateau region and/or favoring the perpendicular orientation of the bicelles in the magnetic field.

Similar observations were made by Dufourc et al. who studied the membrane interaction of melittin and δ-lysin peptides in DPPC vesicles [73,74]. By studying the effect of the peptides on the quadrupolar splittings of DPPC vesicles as a function of temperature, they concluded that the peptide positioning may depend on the physical state of the lipids, the peptide being located at the bilayer surface, capping the polar head group and leading to a greater acyl chain packing, or the peptide being inserted into the bilayer, acting as a spacer and disordering the lipid acyl chains. The temperature dependence of peptide location has also been observed for the human antimicrobial peptide LL-37 by Henzler-Wildman et al., and their results showed the influence of the acyl chain order on the extent of ordering/disordering of the bilayer hydrophobic region by the peptide [75]. In light of these facts, the increase in the $\Delta\nu_{Q(P)}$ may suggest that the 21-mer peptide is located at the bilayer surface of DMPC and DPPC bicelles, caps the lipid molecules, and reduces the lipid acyl chain motion. However, the greater ordering of the DPPC plateau region could be explained by a peptide location closer to the DPPC bicelle interface than in DMPC bicelles. The peptide location in DMPC and DPPC bicelles may be explained by the difference in the bicelle fluidity and temperature. In fact, the ^{2}H NMR spectrum of DMPC bicelle has been recorded at 35 °C, which is 12 °C above the T_m of DMPC lipids, whereas the ^{2}H NMR spectrum of DPPC bicelles has been recorded at the T_m of DPPC lipids. At these temperatures, the DMPC bicelles are then considered to be more fluid than the DPPC bicelles.

3.2.3. Nitrogen-15 NMR

^{15}N NMR spectroscopy is commonly used to determine the membrane topology of peptides and proteins bound to lipid membranes, as well as their rotational diffusion rates. Because of the size and orientation of the σ_{33} ^{15}N chemical shift tensor element, ^{15}N NMR experiments enable the determination of membrane orientation of helical peptides embedded in bilayers aligned with their normal parallel to the magnetic field direction [76,77]. Because the peptide rotational diffusion around the bilayer normal does not change the peptide orientation relative to the magnetic field direction, ^{15}N NMR experiments enable the correlation between the peptide ^{15}N chemical shift and its membrane orientation [78]. Therefore, chemical shifts below 100 ppm or higher than 200 ppm are expected for helical peptides laid at, or perpendicular to the bilayer surface, respectively. In the bilayer perpendicular orientation, however, peptide rotational diffusion introduces a circular distribution of orientations relative to the magnetic field direction, and ^{15}N NMR spectra are sensitive to that motion [79,80]. High-resolution NMR spectra are observed in cases where the rotational diffusion is fast enough to average the CSA, whereas slow rotational diffusion leads to broad resonance line shapes reflecting the peptide orientational distribution [56,78,81]. In addition to rotational diffusion, peptides in interaction with fluid bilayers are also subject to wobbling and vibrational motions that may reduce the ^{15}N CSA. In the present study, the membrane orientation of the ^{15}N-labeled 21-mer peptide has been determined in DMPC bilayers stacked between glass plates, whereas the 21-mer peptide rotational diffusion has been studied in DMPC bicelles.

The ^{15}N NMR spectra of the ^{15}N-labeled 21-mer peptide in the lyophilized state and incorporated into DMPC lipid membranes are displayed in Fig. 4a and b, respectively. The ^{15}N spectrum of the lyophilized 21-mer peptide recorded at ambient temperature is characteristic of a static amide group with a ^{15}N CSA of approximately 185 ppm. When incorporated into DMPC vesicles, the sample

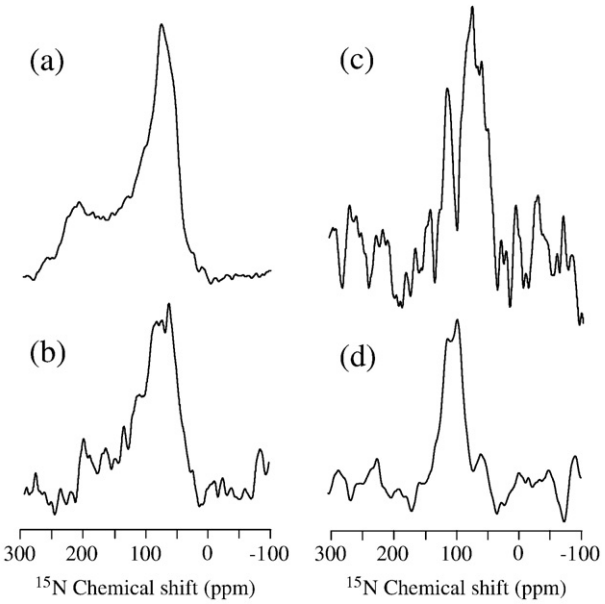

Fig. 4. ^{15}N NMR spectra of the 21-mer peptide in the (a) lyophilized state, (b) incorporated into DMPC multilamellar vesicles at 0 °C, (c) incorporated into DMPC bilayers stacked between glass plates at 37 °C, and (d) in DMPC bicelles at 33 °C. The lipid/peptide molar ratio is 60:1.

temperature has been set at 0 °C to minimize molecular motions that may interfere with an efficient CP. Under such experimental conditions, the amide group exhibits a reduced ^{15}N CSA of approximately 165 ppm. These results indicate that even if the temperature has been lowered to 0 °C, the amide group CSA is partially averaged by molecular motions.

To shed light on the 21-mer peptide orientation and rotational diffusion, we have incorporated the ^{15}N-labeled peptide into DMPC bilayers stacked between glass plates with the bilayer normal parallel to the magnetic field direction and in DMPC bicelles. The ^{15}N NMR spectra are displayed in Fig. 4 (right). As observed on the spectrum of the 21-mer peptide incorporated into oriented DMPC bilayers (Fig. 4c), there is the presence of a main resonance at a chemical shift of 75 ppm, which reflects the 21-mer peptide surface orientation. The width of this resonance suggests however a distribution of orientation of the peptides at the surface of the aligned bilayers. There is also a smaller peak at 120 ppm, which could be related to the presence of a small proportion of disordered lipids in the lipid bilayers oriented between glass plates. In addition to the peptide membrane orientation, we have investigated the peptide rotational diffusion in DMPC bicelles (Fig. 4d) by analyzing the ^{15}N NMR spectral line shape. The ^{15}N NMR spectrum clearly shows the presence of one resonance at a chemical shift of 95 ppm and the absence of a powder pattern characteristic of a peptide circular orientational distribution around the bilayer normal [80,81]. There is also a small peak at the isotropic chemical shift of 120 ppm, which could be related to a small amount of lipids in isotropic structures. The sharp resonance at 95 ppm suggests that the peptide lies on the bicelle planar region and diffuses rotationally around the bilayer normal sufficiently fast to average the ^{15}N CSA. In this case, however, a chemical shift of approximately 140 ppm is expected [78,82], which is far from the value of 95 ppm obtained for the 21-mer peptide in DMPC bicelles. Traaseth et al. observed a similar deviation in the ^{15}N chemical shift of the cytoplasmic domain of the phospholamban peptide that undergoes fast rotational diffusion in lipid bilayers [83]. They attributed the deviation from the expected value of ~140 ppm to the presence of complex dynamic motions in the peptide cytoplasmic domain that, in addition to fast rotational diffusion, may scale the chemical shift value. Alternatively, the peptide may also be localized on the bicelle rim and

oriented perpendicular to the bilayer plane. In such an alignment, the σ_{33} chemical shift tensor element is mainly perpendicular to the magnetic field direction, which would yield to a ^{15}N chemical shift value close to 95 ppm as observed on the spectrum. The increase in chemical shift compared to the expected value of about 75 ppm could in this case be related to an increased overall dynamics of the bicelles.

3.2.4. Carbon-13 NMR

^{13}C CP-MAS experiments under rapid and slow magic-angle spinning have been performed to study the 21-mer peptide structure and dynamics bound to hydrated DMPC multilamellar vesicles at 37 °C. Under MAS conditions, the ^{13}C NMR spectrum is composed of the isotropic resonance flanked by spinning sidebands separated from each other by the MAS frequency, and the number and intensity of spinning sidebands are a function of the MAS speed. The chemical shift of the isotropic resonance is indicative of the peptide secondary structure in which ^{13}CO values of approximately 175.0 and 173.0 ppm are characteristic of α-helical and β-sheet and/or unstructured components [84]. Under slow MAS, several spinning sidebands are present and their number and intensity reflect the size of the peptide carbonyl CSA, which is in turn directly influenced by the ^{13}CO dynamics [85,86]. The ^{13}C CP-MAS NMR spectra of the [^{13}CO]Leu$_{18}$-labeled 21-mer peptide both in the lyophilized state and incorporated to hydrated DMPC vesicles are displayed in Fig. 5.

Prior to the peptide dynamics study, we have performed ^{13}C CP-MAS NMR experiments on the 21-mer peptide in the lyophilized state to determine whether the peptide structure is affected by the lipid environment. As observed on the spectrum of Fig. 5a, there is the presence of two resonances at isotropic chemical shifts of 175.6 and 172.0 ppm, which are characteristic of α-helical and β-sheet and/or unstructured components, respectively. As shown on the ^{13}C CP-MAS NMR spectrum of Fig. 5b, upon the peptide incorporation to hydrated DMPC vesicles at 37 °C and at a lipid/peptide molar ratio of 10:1, the downfield resonance attributed to the α-helical conformation is more intense and pronounced compared to the resonance at 172.0 ppm. These observations suggest that the 21-mer peptide mainly adopts a α-helical conformation in such conditions, as demonstrated previously by circular dichroism experiments [35]. The breath of the 21-mer ^{13}C MAS NMR spectrum obtained in the lipid environment suggests, however, the presence of a small proportion of nonhelical structure. The peptide structure dependence on sample conditions has also been observed for the transmembrane segment of the integral membrane protein phospholamban by Karp et al. [87]. They effectively observed that the peptide exhibits both α-helical and β-sheet or unstructured conformations in nonhydrated PC/PG lipid mixtures, whereas the peptide adopts a α-helical conformation in hydrated PC/PG system.

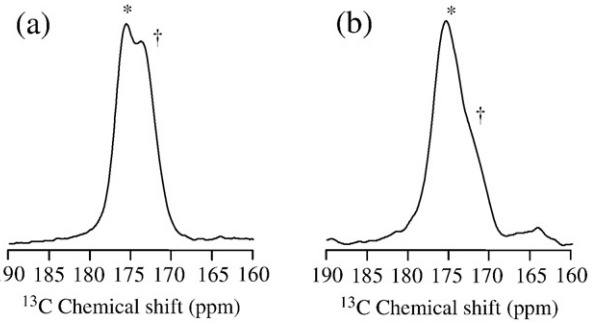

Fig. 5. ^{13}C CP-MAS NMR spectra of the [^{13}CO]Leu$_{18}$-21-mer peptide (a) in the lyophilized state and (b) incorporated to hydrated DMPC vesicles at 37 °C and at a lipid/peptide molar ratio of 10:1. The MAS spinning speed was set at 5000 Hz. The signals marked by * and † indicate isotropic resonance chemical shifts of 175.6 and 172.0 ppm, respectively.

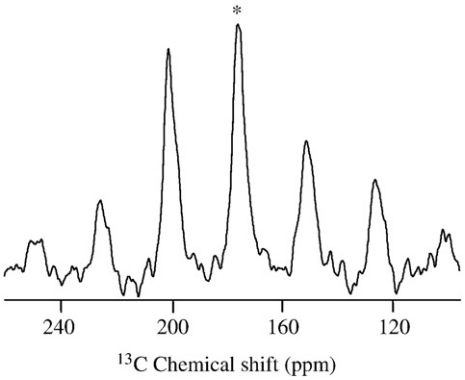

Fig. 6. ^{13}C CP-MAS NMR spectra of the [^{13}CO]Leu$_{18}$-21-mer peptide incorporated to hydrated DMPC vesicles at 37 °C and at a lipid/peptide molar ratio of 10:1. The MAS spinning speed was set at 2500 Hz to generate the spinning sidebands. The signal marked by * indicates the isotropic resonance chemical shift of 175.6 ppm.

Beside the peptide structure study, the ^{13}C CP-MAS NMR experiments under slow MAS give information on the peptide dynamics when bound to fluid lipid bilayers by the analysis of the number and intensity of the spinning sidebands. Such information is essential to the subsequent determination of the peptide structure by the accurate measurement of intramolecular distances [88]. The ^{13}C CP-MAS NMR spectrum of the ^{13}C-labeled 21-mer peptide incorporated to DMPC vesicles at a lipid/peptide molar ratio of 10:1 is displayed in Fig. 6.

As shown in Fig. 6, the number and intensity of spinning sidebands clearly indicate that the 21-mer peptide is immobilized upon its interaction with DMPC bilayers. Such a peptide immobilization may be explained by the peptide aggregation or by binding constraints at a lipid/peptide molar ratio of 10:1. Buffy et al. also observed a similar immobilization of the antimicrobial peptide protegrin-1 incorporated into POPC bilayers at a lipid/peptide molar ratio of 20:1 [89].

3.3. Model of membrane perturbation

The aim of the study was to shed light on the membrane interaction of a 21-mer cytotoxic model peptide that acts as an ionic channel by solid-state NMR spectroscopy. A better understanding of such interactions is the first step involved in the design and synthesis of efficient therapeutic agents possessing specific biological activities, being antimicrobial or cytotoxic. ^{31}P and ^{2}H NMR results on DMPC and DPPC bicelles suggest that the 21-mer peptide stabilizes the bicelle structure. In fact, ^{31}P NMR spectra revealed that the 21-mer peptide increases the temperature range in which bicelles align in the magnetic field. From the ^{31}P resonance line shapes and S_1 order parameter calculation, it seems that the 21-mer peptide does not affect significantly the bicelle mosaic spread at temperatures of bicelle existence. In addition to the ^{31}P NMR, ^{2}H NMR experiments revealed that the 21-mer peptide stabilizes the DMPC and DPPC bicelle hydrophobic core, the effect being more pronounced on DPPC acyl chains. Also, ^{15}N NMR experiments performed in DMPC bilayers stacked between glass plates suggest that the 21-mer peptide lies at the bilayer surface. This model is in agreement with previous attenuated total reflectance experiments, which suggested that the 21-mer peptide is in equilibrium between a major inactive state oriented at the bilayer surface and a minor active transmembrane state [90].

On the other hand, the peptide would undergo fast rotational diffusion around the bilayer normal in DMPC bicelles. A peptide location on the bicelle rim may also be possible considering the chemical shift value of 95 ppm, which suggests that the 21-mer peptide is mainly oriented perpendicular to the magnetic field direction. This hypothesis is also supported by the fact that the peptide length, which is estimated at 31.5 Å, nearly matches the

DMPC bicelle hydrophobic thickness. In such a location on the rim, the 21-mer peptide is expected to displace DHPC molecules, and this may increase the DHPC miscibility into the planar region, as suggested by ^{31}P NMR experiments where the integrity of peptide-containing bicelles was maintained over a larger range of temperatures. This model of membrane perturbation is inspired by the "bicycle tire model" proposed by Segrest to account for the structure of discoidal lipid/apolipoprotein complexes [91]. Dufourcq et al. also used a similar model to explain discoidal particles resulting from the interaction between the melittin peptide and PC bilayers in the gel phase [92]. In such lipid/melittin complexes, peptides are assumed to occupy the bilayer periphery in a parallel or perpendicular orientation relative to the bilayer plane, the latter case being favored since it allows better packing of helices on the rim surface.

4. Conclusions

We have investigated in the present study the membrane interactions and dynamics of a 21-mer cytotoxic peptide incorporated into model membranes by solid-state NMR spectroscopy. Several model membranes were used, namely, DMPC bilayers stacked between glass plates, DMPC multilamellar vesicles, and DMPC and DPPC bicelles. The ^{31}P and ^{2}H NMR results obtained for the peptide-containing bicelles suggest that the 21-mer peptide perturbs the conformation of lipid polar head groups and slightly orders the lipid acyl chains. Also, the 21-mer peptide does not affect the bicelle structure and increases the temperature range in which bicelles align in the magnetic field. On the other hand, ^{15}N NMR experiments on oriented DMPC bilayers stacked between glass plates and DMPC bicelles suggest that the 21-mer peptide adopts a surface orientation and/or is located on the bicelle rim. In addition, ^{13}C NMR experiments performed on the ^{13}C-labeled 21-mer peptide incorporated to DMPC vesicles revealed that the peptide is immobilized upon binding. Based on the NMR results, we propose a model of membrane interaction for the 21-mer peptide where the peptide lies at the bilayer surface, which is the energetically most favorable state, and could adopt a transmembrane orientation under specific conditions of electrochemical and electric gradients [90]. However, the possibility exists that only a minor proportion of peptide units exhibit transmembrane orientation leading to stepwise increases of ion conductivity, and to its cytotoxic activity as previously reported [34,35].

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