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Investigation of the mechanism of action of novel amphipathic peptides: Insights from solid-state NMR studies of oriented lipid bilayers ☆

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ABSTRACT

We have investigated in the present study the effect of both non-selective and selective cationic 14-mer peptides on the lipid orientation of DMPC bilayers by ^{31}P solid-state nuclear magnetic resonance (NMR) spectroscopy. Depending on the position of substitution, these peptides adopt mainly either an α-helical structure able to permeabilize DMPC and DMPG vesicles (non-selective peptides) or an intermolecular β-sheet structure only able to permeabilize DMPG vesicles (selective peptides). Several systems have been investigated, namely bilayers mechanically oriented between glass plates as well as bicelles oriented with their normal perpendicular or parallel to the external magnetic field. The results have been compared with spectral simulations with the goal of elucidating the difference in the interaction of these two types of peptides with zwitterionic lipid bilayers. The results indicate that the perturbation induced by selective peptides is much greater than that induced by non-selective peptides in all the lipid systems investigated, and this perturbation has been associated to the aggregation of the selective β-sheet peptides in these systems. On the other hand, the oriented lipid spectra obtained in the presence of non-selective peptides suggest the presence of toroidal pores. This article is part of a Special Issue entitled: Interfacially Active Peptides and Proteins. Guest Editors: William C. Wimley and Kalina Hristova.

1. Introduction

Bacterial resistance to antibiotics is an important problem due to the abuse and inappropriate use of antibiotics. This important clinical problem has resulted in the appearance of multiresistant bacteria in hospital environments and the increase in the number of infections due to resistant bacteria [1]. The development of antimicrobial agents with novel modes of action is therefore of great importance.

Antimicrobial peptides are ubiquitous in nature and were isolated for the first time in the 1980s from insects and frogs. They were shown to play an important role in these organisms, protecting them from many bacteria in their environment [2,3]. Since then, antimicrobial peptides have been found in a wide variety of organisms, from mammals to bacteria, plants and fungi [4,5]. This variety reflects the importance of antimicrobial peptides in the innate immune system of these organisms [6]. Some antimicrobial peptides can also have antiviral, antifungal and anticancer properties [7].

Most antimicrobial peptides share common characteristics: they are relatively short (12 to 100 amino acids), positively charged (net charge ranging from +2 to +9), and their three-dimensional structure is amphiphilic [8]. Their overall positive charge plays in turn an important role in their selectivity for negatively charged membranes, such as bacterial membranes. Using one or more of these characteristics as a starting point, many research groups are trying to reproduce the effects of natural antimicrobial peptides by designing varied synthetic peptides.

A 14-mer peptide, designed in our laboratory, is made of 10 leucine residues (L) and 4 phenylalanine residues modified by the addition of crown ethers (CE) (Fig. 1A). The nature of these residues, but also their sequence (Fig. 1A), result in this short peptide adopting mainly an α-helical secondary structure. In this amphiphilic structure, the CE are close to each other and localized on the same side relative to the main axis of the helix.

The base 14-mer peptide was studied extensively in our laboratories [9,10]. Its antimicrobial potential and membrane disruptive activity were first introduced by Vandenberg et al. [11] and then studied in depth by Ouellet et al. using various spectroscopic techniques [12,13]. The antimicrobial nature of the 14-mer peptide, however, is associated with a significant hemolytic character. In addition, its overall neutral charge prevents it from selectively targeting bacterial membranes. A systematic approach was therefore used to replace one, two or three of each leucine residues by positively charged amino acids, namely lysine or arginine residues. More than 300 cationic derivatives of the base 14-mer peptide were synthesized and named according to the position of the replaced leucine residue [14] (some examples are represented in Fig. 1B).

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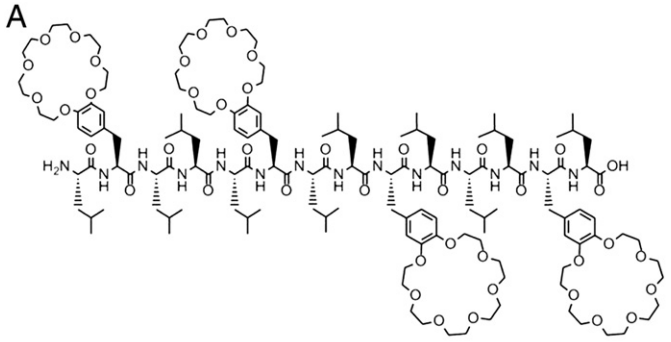

Fig. 1. (A) Sequence of the neutral base 14-mer peptide and (B) examples of positively charged analogs used in this study.

We have previously investigated using fluorescence and infrared spectroscopy the interactions of cationic 14-peptides in which one or two leucines were substituted by positively charged lysine residues at each position [15], as shown schematically in Fig. 1B. Our results demonstrated that the position of substitution dictates the peptide selectivity, with selective peptides causing calcein release only with negatively charged dimyristoylphosphatidylglycerol (DMPG) membranes, mimicking bacterial membranes, while non-selective peptides induce calcein release with both negatively charged model membranes and zwitterionic dimyristoylphosphatidylcholine (DMPC) membranes, mimicking eukaryotic membranes, as shown in Table 1. In addition, our results showed that non-selective peptides adopt an α-helical structure while an intermolecular β-sheet structure is observed for selective peptides [15] (Table 1). We have also used a combination of ^{31}P and ^{2}H solid-state nuclear magnetic resonance (NMR) spectroscopy to determine how the peptide interacts with the lipid headgroups and acyl chains, respectively [16]. ^{2}H NMR and FTIR results reveal an ordering of the hydrophobic core of bilayers when leakage is noted, i.e. for DMPG vesicles in the presence of both type of peptides and DMPC vesicles in the presence of non-selective peptides. However, selective peptides have no significant effect on the ordering of DMPC acyl chains. The ability of these 14-mer peptides to permeabilize lipid vesicles therefore appears to be in part related to their ability to increase the order of the bilayer hydrophobic core. However, an understanding on how the peptides affect the lipid orientation is of primary importance to fully elucidate their mechanism of action. More specifically, the formation of toroidal pores will result in a local change of lipid orientation while the lipid orientation will not be affected in the presence of barrel-stave type pores [7,17].

The goal of the present study was therefore to investigate by ^{31}P solid-state NMR spectroscopy the effect of both non-selective and selective cationic 14-mer peptides on the lipid orientation in DMPC bilayers in order to explain the difference in selectivity of these two types of peptides in zwitterionic membranes. Several systems have been investigated, namely bilayers mechanically oriented between glass plates as well as bicelles oriented with their normal perpendicular or parallel to the external magnetic field. The results have been compared to spectral simulations with the goal of elucidating the difference in the interaction of these two types of peptides with zwitterionic lipid bilayers.

2. Materials and methods

2.1. Materials

DMPC, dihexanoylphosphatidylcholine (DHPC) and 1-myristoyl-2-[4-(4-biphenyl)butanoyl]-*sn*-glycero-3-phosphocholine (TBBPC) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without purification. HEPES, EDTA, NaCl and KCl were purchased from Sigma-Aldrich (St-Louis, MO). Water used for buffer preparation was distilled and deionized using a Barnstead NANOpurII system (resistivity of 18.2 MΩ/cm; Boston, MA) with four purification columns. All solvents were of reagent grade or HPLC grade quality, purchased commercially and used without any further purification. Fmoc-protected amino acids were purchased from Matrix Innovation (Québec, QC, Canada). All other chemicals were of reagent grade. Glass cover slides of 0.13–0.17 mm thickness were purchased from VWR Scientific (West Chester, PA) and cut into 9 mm × 22 mm rectangles.

2.2. Peptide synthesis

The 14-mer peptides were prepared by solid-phase synthesis as described previously [15] using Wang resin as solid support and N-Fmoc-protected amino acids.

2.3. Sample preparation

2.3.1. Lipid bilayers oriented between glass plates

The DMPC bilayers oriented between glass plates were prepared by dissolving 5 mg of phospholipids in 100 μL of chloroform, and the solution was deposited onto 15 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. This procedure yielded satisfactory alignment of the membrane, as indicated by the narrow ^{31}P resonances of the pure lipid bilayers. For the preparation of the peptide-containing bilayers, the dry peptide was co-dissolved with dry lipids in chloroform/methanol 50/50 v/v at a lipid/peptide molar ratio of 20:1. The following steps are the same as described above for the preparation of pure aligned bilayers stacked between glass plates.

2.3.2. DMPC/DHPC and TBBPC/DHPC bicelles

DMPC/DHPC [18,19] and TBBPC/DHPC [20–24] bicelles were prepared by mixing the desirable quantity of DMPC or TBBPC dissolved in chloroform and DHPC also dissolved in chloroform. The total mass of phospholipids was 20 mg and the long-chain (DMPC or TBBPC):short-chain (DHPC) phospholipid molar ratio (q) was 3.5 for DMPC/DHPC bicelles and 6.7 for TBBPC/DHPC bicelles. A suitable amount of peptide dissolved in $CHCl_3$/MeOH (1/1 v/v) was added in the lipid solution to obtain a phospholipid/peptide molar ratio of 60:1. The residual solvent

Table 1
Peptides investigated in the present study.

Name	Secondary structure	Selectivity towards negatively charged lipid bilayers	Effect on oriented lipid bilayers
K3	α-helix	Non-selective	Pore formation
K5K10	α-helix	Non-selective	Pore formation
K4	Aggregated β-sheets	Selective	Lipid aggregation
K4K11	Aggregated β-sheets	Selective	Lipid aggregation

was removed under a stream of nitrogen gas, followed by an overnight lyophilization. The dried samples were hydrated with 80 μL of HEPES 100 mM, EDTA 5 mM (pH 7.4) and 100 mM NaCl (for TBBPC/DHPC bicelles) or 100 mM KCl (for DMPC/DHPC bicelles), giving a total proportion of 20% (w/w) lipids in buffer. The lipid suspensions were then centrifuged at 6500 rpm for 5 min. Preparation of bicelles and TBBPC bicelles was ensured by repeating at least 3 cycles of vigorous vortexing, freezing (liquid N_2), thawing (50 °C), vortexing, and centrifuging (6500 rpm for 5 min). Then, an additional cycle was performed by submitting the samples to a last freeze followed by thawing at ambient temperature before packing the samples into 4 mm NMR tubes prior to data acquisition.

2.4. ^{31}P NMR experiments

Proton-decoupled ^{31}P solid-state NMR spectra were acquired with a Bruker Avance 400 MHz spectrometer (Bruker Biospin, Milton, Ontario, Canada). The thin glass plates were inserted into a flat coil of a home-built solid-state NMR probehead with the glass plate normal oriented parallel to the magnetic field direction. The bicelle spectra were acquired by placing the samples into a 4-mm NMR tube inserted into a magic-angle spinning (MAS) probe (Bruker Biospin, Milton, Ontario, Canada). The spectra were obtained at 161.9 MHz with a Hahn echo sequence [25] and TPPM proton decoupling [26]. Using 4096 data points, the spectra were acquired with a pulse length of 4.5 μs and a recycle delay of 4 s. The spectral width was 50 kHz and a line broadening of 50 Hz was applied to all spectra. 3000 scans were acquired for the glass-plates oriented-sample spectra while the bicelle spectra were acquired using 1200 scans. The chemical shifts were referenced relative to external H_3PO_4 85% (0 ppm). Spectral simulations were performed using the MATLAB software (The MathWorks Inc., Natick, MA).

3. Results and discussion

Since the phospholipid headgroup contains a phosphorus-31 nucleus with a 100% natural isotopic abundance, ^{31}P NMR is a powerful technique to monitor changes occurring in the polar region of the bilayer [27–29]. We have previously used ^{31}P solid-state NMR on multilamellar vesicles to determine the influence of the base 14-mer peptide [12] as well as different non-selective and selective cationic 14-mer peptides on the dynamics of the lipid polar head group and the morphology of the lipid vesicles [16]. However, this technique does not allow us to precisely determine the presence and nature of the deformations induced by the peptide. The effect of both non-selective and selective peptides on the orientation of phospholipid molecules was first investigated in bilayers oriented between glass plates [13,30–35]. As detailed below, these systems allow to detect membrane deformations that are often short-lived in multilamellar vesicles and therefore, difficult to detect.

3.1. Lipids macroscopically oriented between glass plates

There are notable differences between lipids macroscopically oriented between glass plates and multilamellar vesicles. Indeed, the lowest hydration and the presence of electrostatic interactions between the glass plates and the first layers of lipids decrease the fluidity of these model membranes and the lipid rate of motions. These phenomena help to stabilize membrane deformations, if any, and thus make them detectable by NMR [30,31,34,35]. Also, used in conjunction with spectral simulations, it becomes possible to determine the nature and shape of the deformations induced by the peptides [34]. This behavior has been exploited previously in studies of the base neutral 14-mer peptide, and the results suggest that this peptide is adsorbed to the surface of the bilayer and imposes a bending stress on the membrane due to its amphiphilic character [13]. This constraint would lead ultimately to the formation of toroidal pores.

We have therefore used ^{31}P solid-state NMR of macroscopically oriented DMPC samples to detect the presence of deformation in the phospholipid bilayers upon the addition of both non-selective and selective cationic 14-mer peptides. For perfectly aligned lipid bilayers oriented with their normal parallel to the magnetic field, the NMR spectrum consists of a single peak at a chemical shift equal to $\delta_{//}$ (Fig. 2A). The addition of a membrane disruptive peptide to the system can change the orientation of some of the phospholipid molecules and additional signals will be detected at lower chemical shifts. These signals can take several forms and the ratio between the latter and the peak at $\delta_{//}$ can vary greatly. The presence of this deformation is shown in Fig. 2B after adding the peptide K3, a non-selective peptide, to DMPC bilayers oriented between glass Plates. A lipid-to-peptide molar ratio of 20:1 was used in this case to increase the intensity of the signal resulting from peptide perturbation but a perturbation was also visible at a lipid-to-peptide molar ratio of 60:1 (results not shown).

We have also compared the effects of two non-selective peptides (K3 and K5K10) and of two selective peptides (K4 and K4K11) on the orientation of DMPC bilayers mechanically oriented between glass plates (Fig. 3) [36]. The results presented in Fig. 3 and summarized in Table 1 indicate that the addition of both non-selective and selective peptides induces a loss of orientation, with a greater effect observed with the selective peptides. More specifically, the non-selective peptides induce the appearance of a powder pattern between about 20 ppm and −20 ppm that is attributed to the presence of a small proportion of non-oriented lipids. A small peak is also present at around 20–25 ppm in the spectra in the presence of peptides, that could be attributed to membrane thinning [34]. The results obtained with the non-selective peptides are similar to those obtained with the base 14-mer peptide [13], indicating that peptides structured as α-helices disturb DMPC bilayers in a similar manner, whether they contain a positive charge or not. In addition, increasing the number of positive charge by one does not exacerbate the perturbation induced by the peptides in interactions with zwitterionic model membranes. Therefore, the secondary structure of the peptides appears to be the driving force in their membrane interactions.

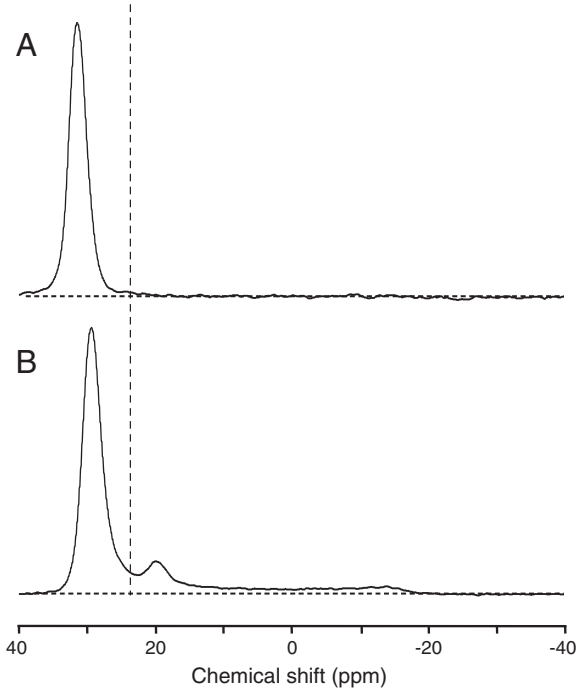

Fig. 2. ^{31}P NMR spectra of DMPC bilayers macroscopically oriented between glass plates in (A) the absence and (B) the presence of the peptide K3 (lipids/peptide molar ratio of 20:1). The measurements were performed at 37 °C. The signals located to the right of the dashed line are due to membrane perturbations.

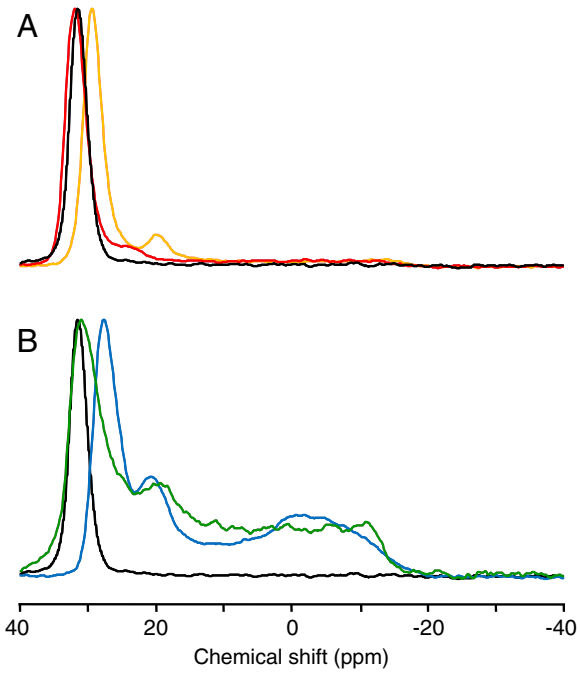

Fig. 3. ^{31}P NMR spectra of DMPC bilayers macroscopically oriented between glass plates (A) in the absence (black) and presence of two nonselective peptides, K3 (yellow) and K5K10 (red); and (B) in the absence (black) and presence of two selective peptides, K4 (green) and K4K11 (blue). The measurements were performed at 37 °C and at a lipid/peptide molar ratio of 20:1.
Adapted from [36] and reproduced with permissions.

The greater effect observed with the selective peptides is surprising since these peptides do not induce calcein release in DMPC vesicles [15]. Although the presence of phospholipids whose orientation was disrupted reflects the membrane activity of this peptide, it would be useful to know the nature of the deformation.

3.2. Spectral simulations

The spectral simulation approach proposed by Wi & Kim has been applied to two types of membrane deformation: toroidal pores and thinning in the membrane [34]. These two changes are relevant in the study of cationic 14-mer peptides as both were previously suggested to occur in the presence of the base 14-mer peptide [13]. An analysis of the spectra shown in Fig. 3 was therefore performed by spectral simulations (Fig. 4A and B). These were optimized to reproduce the signals between about 30 and −15 ppm in order to test the hypothesis of toroidal pores previously suggested for the base 14-mer peptide. A spectrum with a pore radius of 10 Å, based on the hydrodynamic radius of calcein is 8 Å [37], is able to adequately simulate the broad spectral feature observed in the presence of the non-selective K3 peptide (Fig. 4A). Simulations with other pore sizes (results not shown) indicate however that it would be difficult to precisely determine the exact toroidal pore size only by comparing these spectra. These simulations also allow us to observe in all cases a very clear separation between the peak at $\delta = \delta_{//}$ and the small contribution at $\delta \approx 20$ ppm. The latter cannot be explained by a pore or an ellipsoid given its position. It is also unlikely that these signals result from more mobile phospholipids (with smaller CSA) due to the absence of intermediate signals. Membrane thinning effectively reproduces the shape of the peak [34], suggesting that a small

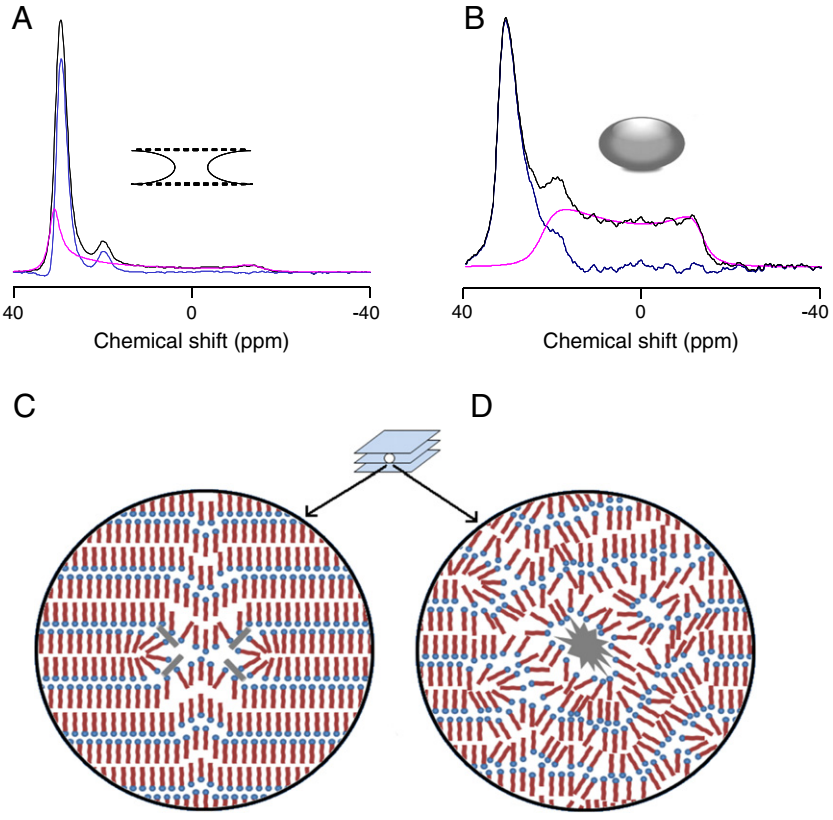

Fig. 4. (A) ^{31}P NMR spectrum of DMPC bilayers macroscopically oriented between glass plates in the presence of the non-selective peptide K3 (lipid/peptide molar ratio of 20:1) (black) and simulated spectra (purple) of toroidal pores with a radius of 10 Å. (B) ^{31}P NMR spectrum of DMPC bilayers macroscopically oriented between glass plates in the presence of the selective peptide K4 (lipid/peptide molar ratio of 20:1) (black) and simulated spectrum (purple) of an ellipsoid deformation. The spectra resulting from the subtraction of simulated spectra are presented in blue. The shape of the simulated deformation is also represented in each case. Schematic representation of disorder induced by (C) pore formation and (D) peptide aggregation in oriented bilayers.
Panels C and D adapted from [36] and reproduced with permissions.

proportion of lipids could experience membrane thinning upon interactions with non-selective peptides.

The simulations presented in Fig. 4B clearly indicate that the interaction of selective peptides with oriented bilayers does not result into pore formation since an ellipsoid simulation [38] better reproduces the shape of the signal detected between 20 and −20 ppm. These results therefore suggest the absence of toroidal pores in this sample, which is in agreement with fluorescence studies indicating that these selective peptides do not induce leakage in DMPC vesicles [15].

3.3. Comparison between non-selective and selective peptides

The experimental results and simulations presented above suggest that selective peptides do not induce pore formation as non-selective peptides do (Fig. 4C) but rather aggregate at the surface of the membrane, affecting the lipid organization in a greater manner, as illustrated schematically in Fig. 4D [36]. This result is in agreement with a study by Jean-Francois et al. [39] that suggested that multimerization of cateslytin peptides at some regions of the bilayer surface induces an ordering of lipid acyl chains but without pore formation. Leakage was then explained by membrane defects induced by the thickness difference between free and peptide-associated membrane regions. A similar hypothesis was also proposed to explain leakage induced by two alpha/beta antimicrobial peptides [40]. Therefore, the mechanism of action of selective cationic 14-mer peptides could involve the formation of domains of higher order in which permeabilization would be due to defects at the domain borders.

3.4. DMPC/DHPC and TBBPC/DHPC bicelles

We have also investigated the effect of the non-selective peptide K5K10 and of the selective peptide K4K11 on the orientation of lipids in two types of bicelles, namely DMPC/DHPC bicelles oriented with their normal perpendicular to the external magnetic field and TBBPC/DHPC bicelles oriented with their normal parallel to the external magnetic field. These two types of bicelles have proven to be useful for the investigation of the structure and interactions of peptides in membranes [18,21,41–44].

The results are first presented in Fig. 5 for DMPC/DHPC bicelles oriented with their normal perpendicular to the external magnetic field for two peptides. The pure bicelle spectrum consists of two peaks that have been attributed to lipids on the planar section (larger peak) and the torus section (smaller peak) of the bicelles [18,38]. The results indicate that the addition of the non-selective peptide K5K10 results in only a small shift of the peak associated with the planar section of the bicelle, suggesting that the peptide does not significantly perturb the bicelle orientation. The small increase in chemical shift might be associated with a small increase in the headgroup dynamics in the planar section of the bicelles. It is also interesting to note that the intensity of the peak associated with the edge section of the bicelles is slightly increased in the presence of the non-selective peptide K5K10, which might suggest a preferential association of this peptide with the bicelle edges. On the other hand, the addition of the selective peptide K4K11 results in a more significant change in the bicelle spectrum, with the shift of the peak associated with the torus section of the bicelle towards the isotropic chemical and a significant broadening of the peak associated to the planar section. This result indicates that, as observed in lipid bilayers oriented between glass plates, the selective peptide has a greater effect on the lipid orientation. This therefore indicates that the secondary structure of the peptide is also important in the perturbation of lipid orientation in bicelles, with the α-helical peptides having less effect than β-sheet aggregated peptides.

The results obtained with TBBPC/DHPC bicelles are presented in Fig. 6. The TBBPC molecule has one aliphatic chain that contains a biphenyl unit. This phospholipid therefore has an intrinsic large positive diamagnetic susceptibility $\Delta\chi$ which induces a spontaneous orientation of the bicelles in a magnetic field with their normal parallel to the magnetic field (smectic orientation) [20,24]. The spectrum of the pure bicelle system is similar to that observed for the DMPC/DHPC bicelle system, namely with two peaks that can be attributed to lipids in the planar and torus section of the bicelles. However, the chemical shift are positive and about twice the values observed in DMPC/DHPC bicelles, due to the parallel orientation of the lipids in TBBPC/DHPC bicelles. The

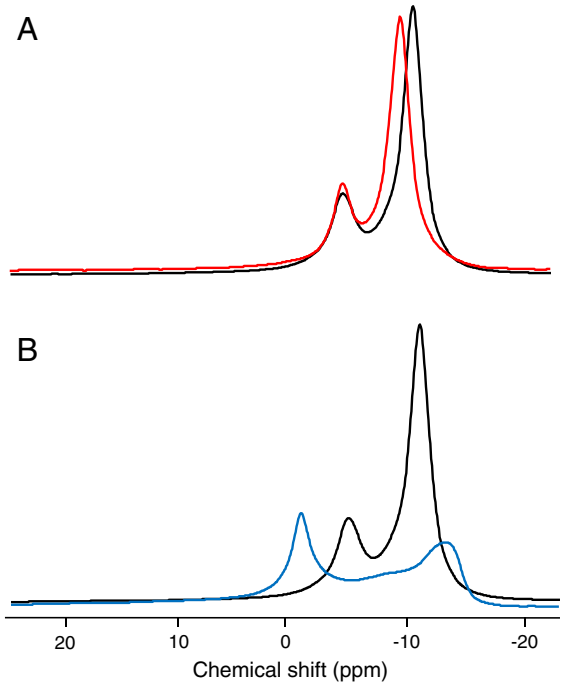

Fig. 5. ^{31}P NMR spectra of DMPC/DHPC (3.5:1) bicelles (A) in the absence (black) and presence of a nonselective peptide, K5K10 (red); and (B) in the absence (black) and presence of a selective peptides, K4K11 (blue). The measurements were performed at 37 °C and at a lipid/peptide molar ratio of 60:1.

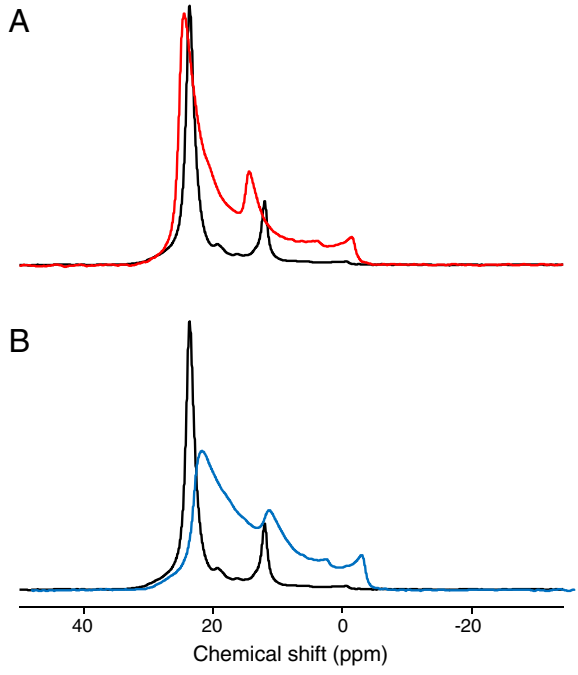

Fig. 6. ^{31}P NMR spectra of TBBPC/DHPC (6.7:1) bicelles (A) in the absence (black) and presence of a nonselective peptide, K5K10 (red); and (B) in the absence (black) and presence of a selective peptides, K4K11 (blue). The measurements were performed at 37 °C and at a lipid/peptide molar ratio of 60:1.

addition of the non-selective peptide K5K10 results in a small broadening of both resonances and the appearance of a broad powder pattern whose shape can be associated to toroidal pore formation, as observed for lipids oriented between glass plates. On the other hand, the addition of the selective peptide K4K11 results in a much greater broadening of the two bicelle resonances and the appearance of a more significant powder pattern, again reminiscent to the results obtained with lipids oriented between glass plates.

The results presented above are particularly important as they demonstrate that the peptide-induced perturbations observed in lipids oriented between glass plates are also observed in the more fluid bicelle systems. These results also clearly demonstrate that both types of bicelles could be used for the study of the structure of non-selective α-helical peptides. However, the large perturbation induced by the β-aggregated peptides could prevent the use of bicelles for their structural study by solid-state NMR.

4. Conclusions

We have investigated in the present study the effect of cationic peptides on the orientation of lipids in different oriented systems, namely lipids mechanically oriented between glass plates as well as bicelles oriented with their normal perpendicular and parallel to the external magnetic field. The results indicate that the secondary structure of the peptides, and hence their selectivity towards bacterial membranes, greatly influence the types of deformation that these peptides induce in oriented lipid systems. More specifically, non-selective peptides, that adopt an α-helical structure, induce a smaller perturbation of lipid orientation, in agreement with the formation of toroidal pores, while selective peptides, that adopt an aggregated β-sheet structure, induce a much greater perturbation of lipid orientation. These perturbations have been confirmed by ^{31}P spectral simulations. These results are of primary importance for the understanding of the mechanism of action of these novel cationic amphipathic peptides. In addition, they provide insights for the use of oriented lipid systems in the study of the structure of membrane-associated peptides.

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