Review

Biophysical studies of the interactions between 14-mer and 21-mer model amphipathic peptides and membranes: Insights on their modes of action

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Abstract

We have investigated the interactions between synthetic amphipathic peptides and zwitterionic model membranes. Peptides with 14 and 21 amino acids composed of leucines and phenylalanines modified by the addition of crown ethers have been synthesized. The 14-mer and 21-mer peptides both possess a helical amphipathic structure as revealed by circular dichroism. To shed light on their mechanism of membrane interaction, different complementary biophysical techniques have been used such as circular dichroism, fluorescence, membrane conductivity measurement and NMR spectroscopy. Results obtained by these different techniques show that the 14-mer peptide is a membrane perturbator that facilitate the leakage of species such as calcine and Na ions, while the 21-mer peptide acts as an ion channel. 31P solid-state NMR experiments on multilamellar vesicles reveal that the dynamics and/or orientation of the polar headgroups are greatly affected by the presence of the peptides. Similar results have also been obtained in mechanically oriented DLPC and DMPC bilayers where different acyl chain lengths seem to play a role in the interaction. On the other hand, 2H NMR experiments on multilamellar vesicles demonstrate that the acyl chain order is affected differently by the two peptides. Based on these studies, mechanisms of action are proposed for the 14-mer and 21-mer peptides with zwitterionic membranes.

Keywords: Synthetic amphipathic peptide; Model membrane; NMR spectroscopy; Circular dichroism; Single-channel conductivity; Fluorescence

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1. Introduction

In the last few decades, bacterial resistance against commonly used antibiotics has become an increasingly important public health problem. Enormous efforts are devoted to reduce the number of antibiotic resistant bacteria. Scientists have therefore concentrated their efforts on the elucidation of bacterial cell functions to identify new bacterial targets, and on the design and development of new classes of antimicrobial agents that possess novel modes of action that could overcome known mechanisms of bacterial resistance [1–5]. The focus of the present review is on the design and synthesis of novel potential antimicrobial compounds.

More than 880 different antimicrobial peptides, which are an important component of the defense system of a variety of species such as mammals, plants, insects, viruses and bacteria, have been reported or predicted from nucleic acid sequences and they became the focus of many researches [5–7]. Despite the fact that natural antimicrobial peptides do not show primary structure homology, they possess the following general trends: they are relatively small, they have a net positive charge of +2 to +7, they adopt a variety of three dimensional structures such as α-helix and β-sheet conformations conferring an amphipathic character, they possess a broad spectrum of activity and they are recognized to kill bacteria rapidly so they do not contribute to the rapid emergence of resistance [6,8–12].

Antimicrobial peptides as a source of potential therapeutic agents have brought the scientific community to shed light on the structural parameters required for their antimicrobial activity, in an ultimate goal of elucidating their mechanisms of action. A better understanding of these mechanisms will allow the design of novel therapeutic peptides that possess the required specificity against bacteria and that act via new modes of action defying most of the known mechanisms of bacterial resistance [5]. Despite the characteristics listed above, several natural antimicrobial peptides such as melittin from bee venom present the disadvantage of being toxic to eukaryotic cells [13]. Many research groups have therefore oriented their work on the design and synthesis of model antimicrobial peptides with increased antimicrobial activity and reduced toxicity to eukaryotic cells. The synthetic peptide approach in a view of examining variations in structural parameters such as hydrophobicity/hydrophilicity balance, amphipathicity, charge and helicity will enable rapid progress in the design of selective antimicrobial peptides in an efficient way [14]. These studies have brought new information on the structural parameters required for the desired antimicrobial activity, and general mechanisms of action that follow from such parameters have been suggested, such as the “carpet-like”, “barrel-stave” and “toroidal” mechanisms [15–20].

For example, Stark et al. have designed a new category of nonamphipathic hydrophobic antimicrobial peptides that seem to selectively permeabilize bacterial cells via the “carpet-like” mechanism [21]. From their study, they concluded that the antimicrobial activity coincides with a threshold hydrophobicity that is required for peptide insertion into bacterial membranes. A 26-residue peptide with varying D- and L-amino acids in the primary sequence has been studied by Chen et al. to investigate the effect of peptide hydrophobicity/hydrophilicity balance, amphipathicity and helicity on biological activity [14]. Synthetic peptides with ingenious conformation named antimicrobial dendrimeric peptides, that can be easily chemically modified to amplify the antimicrobial activity, have been synthesized by Tam et al. [22]. By varying the size of the dendrimeric peptides, they have obtained an optimal peptide with four branched lysine residues that adopts a barrel-like structure with high antimicrobial activity without hemolysis. Many research groups have focused their attention on hybrids of natural antimicrobial peptides such as, for example, the cecropin-melittin hybrids [23–26]. By joining specific amino acid sequences from natural antimicrobial peptides, they have obtained very promising hybrids with improved antimicrobial activity without hemolytic properties. Schmitt et al. and Epand et al. have both oriented their research on the design and structure–function study of synthetic peptides composed of unnatural β-amino acids called β-17 peptides and an alternance of α- and β-amino acids called αβ-peptides [27–29]. Variation in the helical conformation of these foldamers confers different biological activities that can be correlated to the amphipathic character of the helical structure. The membrane permeabilization activity of the peptides listed above has been tested on model membranes and on the membranes of both Gram-negative and Gram-positive bacteria. These studies have revealed that the lipid membrane of prokaryotic and/or eukaryotic cells seems to be the primary or final target of such peptides and that their biological activity appears to be modulated by peptide structural parameters such as helicity, positive charge, amphipathic character and hydrophobicity [30–34].

Another interesting point to be addressed here is the design of ion channel peptides. Many natural antimicrobial peptides acting via a channel mechanism and pore-forming helical peptides are reported in the literature [35]. Ion channel peptides are promising antimicrobial agents since they could affect the bacterial membrane electrochemical potential and then kill the pathogens [36]. Many research groups and in particular DeGrado et al. have elucidated the structural parameters required for membrane channel activity and they designed model ion channel peptides [37–39]. The model developed by DeGrado is a 21-residue peptide composed of leucine and serine residues. This peptide was shown to form ion channels and showed ion permeability and open lifetime properties similar to the acetylcholine receptor [40–42]. Fernandez-Lopez et al. have also designed cyclic D,L-α-peptides able to selectively target and self-assemble in bacterial membranes to exert antibacterial activity by increasing membrane permeability [43]. For a detailed list of synthetic model ion channel peptides, the readers are referred to the paper of Gokel et al. [39].

2. Model amphiphatic peptides containing crown ethers

Inspired by the promising future of synthetic antimicrobial peptides as an alternative to antibiotics, the research group of N. Voyer has concentrated its efforts on the design and synthesis of novel model peptides presenting various membrane activities.
The general idea was to use an α-helical peptidic framework made from leucines and phenylalanines modified with crown ethers \[44\]. These amino acids are known to increase the helical propensity of the chain and crown ethers are ingeniously positioned to lead to their alignment under a helical conformation. This organization is useful for two reasons. First, once incorporated into membranes, the peptide can allow ion translocation from one crown ether to another and thus create a channel \[45,46\]. Second, the polar crown ethers on the same side and the hydrophobic leucines on the other side result in an amphipathic peptide that can mimic properties of natural membrane-active peptides \[47\]. A 21-mer peptide was first designed, since it has the required length to fit in biological membranes and it could therefore act as an ion channel. In addition, a 14-mer peptide was designed as a lytic peptide that could more easily disturb membranes because of its shorter length not matching a membrane thickness. These peptides were prepared by solid-phase peptide synthesis on oxime resin, a technique that has been shown to be fast and efficient \[48,49\]. This strategy allows easy modifications at the N- and C-termini by peptidic coupling or nucleophilic cleavage to rapidly obtain a wide variety of analogues \[50,51\]. Protected and fully deprotected compounds have been synthesized, and other peptides bearing more or less polar terminal groups were also prepared (Fig. 1). Unless indicated otherwise, the peptides investigated in the present study are the 14-mer peptide with \(n=2\) and \(Y=\text{OH}\) (peptide B) and the 21-mer peptide with \(n=3\) and \(Y=\text{OCH}_3\) (peptide C).

Since the biological membrane seems to be the target of several natural and synthetic antimicrobial peptides, the structure and function of the 14-mer and 21-mer peptides have been investigated in synthetic model membranes. The advantage of model lipid bilayers resides in the fact that the physical state as well as the chemical composition of these membranes can be easily modified using zwitterionic and/or anionic lipids to mimic eukaryotic and bacterial membranes. Lipid vesicles with different sizes can also be prepared. Multilamellar vesicles with typical sizes of the order of a micron were used for NMR studies \[52\], while lipid vesicles with sizes between 175 and 200 nm were prepared via extrusion and filtration with micro-pore filters for leakage studies \[53\].

Notably, unlike most known natural and synthetic antimicrobial peptides, the 14-mer and 21-mer peptides used in the present study do not possess a net positive charge. Their neutral nature allows the study of the effect of hydrophobic forces in the interaction with lipids without the influence of strong electrostatic interactions.

3. Conformation of the 14-mer and 21-mer peptides

Circular dichroism (CD) spectropolarimetry was used to investigate the conformation of the 14-mer and the 21-mer peptides both in solution and bound to lipid bilayers. Measurements were performed in trifluoroethanol with a peptide concentration of 1.0 mg/mL and in egg yolk lecithin vesicles with a lipid-to-peptide molar ratio of 80:1. In the latter case, the lipids and peptides were codissolved in chloroform prior to sample hydration. The specific dichroic signatures of conformations such as \(\alpha\)-helix, \(\beta\)-sheet and random coil are well documented. For \(\alpha\)-helices, two minima at 208 and 222 nm are expected, for \(\beta\)-sheets a minimum at 215 nm is observed, while the random coil conformation gives a minimum

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Fig. 1. 7-mer, 14-mer and 21-mer peptides with different N-terminal and C-terminal groups. Unless indicated otherwise, the peptides investigated in the present study are the 14-mer peptide with \(n=2\) and \(Y=\text{OH}\) (peptide B) and the 21-mer peptide with \(n=3\) and \(Y=\text{OCH}_3\) (peptide C).
at 195 nm and a maximum at 220 nm. Fig. 2 displays the CD spectra of the 14-mer and the 21-mer peptides both in TFE and in lipid vesicles. All CD spectra revealed that the 14-mer and 21-mer peptides exhibit an α-helical conformation in both media. An interesting point to note here is the partial disappearance of the component at 209 nm on the CD spectrum of the 21-mer peptide in vesicles (Fig. 2). It has already been reported that the extent of this disappearance varies with the cell path length at constant concentration. A similar CD behavior has already been reported in the literature for α-helical peptides incorporated in lipid bilayers. This phenomenon has been suggested to be associated with the orientation of the helical peptides in the lipid bilayer on the quartz surface [54]. CD studies at different concentrations (0.1 μM–0.1 mM) also point out that the 21-mer peptide and its analogues do not tend to aggregate in low polarity environment as suggested by the conservation of the ellipticity at 222 nm [46].

4. Transport and lytic activity of the 14-mer and 21-mer peptides

To investigate the transport and lytic properties of the crown peptides, different complementary techniques have been used such as pH-stat, monomolecular membrane conductivity, 23Na NMR and calcein fluorescence spectroscopies.

4.1. pH-stat

The pH-stat technique is based on the pH difference between the internal and external environment of unilamellar lipid vesicles [55]. Essentially, vesicles made with an internal pH of 6.6 are placed in an external solution of pH 7.6. Then specific ions and FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) [56], a proton carrier, are added and transport ability can be monitored by the release of protons required to maintain the electroneutrality across the vesicle membranes [57]. Results obtained in PC/PA/cholesterol vesicles with the 21-mer peptide indicate a similar Cs+ transport activity as gramicidin A, a peptide known to form very efficient ion channels [44]. Moreover, the 21-mer peptide showed comparable activities with Li+, Na+, K+ and Rb+, while analogues without crown ethers and the monomer of a crown ether phenylalanine showed only very weak transport typical of a carrier mechanism. These results suggest that the 21-mer peptide affects ion transport via a channel mechanism and that ions most probably pass through the crown ether stack instead of through a pore formed by self-assembled peptides. On the other hand, the addition of Triton X-100 at the end of every experiment led to the complete lysis of the vesicles and the release of entrapped H+, which demonstrates that the vesicles are still intact at the end of the assay and that the 21-mer peptide does not act as a surfactant.

4.2. Monomolecular membrane conductivity

To further characterize the transport ability of the 21-mer peptide, single-channel measurements [58,59] have been performed using a modified patch clamp technique [60]. This technique is based on the measurement of the electric current resulting of ions passing through a diphytanoylglycerolphosphocholine (DiPhy) bilayer adsorbed on a pipette tip. Results with the 21-mer peptide in the presence of K+ show typical transitions (steps) between “closed” and “open” states, that are characteristic of ion channel activity observed with natural channel proteins [45,46]. In DiPhy membranes, the 21-mer peptide has a current level of 2.5±0.2 pA with an average lifetime for the open states of about 1 s. Furthermore, experiments with the 21-mer peptide bearing smaller 18-crown-6 moiety showed a conductivity of 3.5±0.2 pA and average lifetime of opening of 100-200 ms, differing greatly from results obtained with 21-crown-7 channel and supporting that ions travel across the crown ether stack (J.-C. Meillon, N. Voyer, unpublished results). These results agree with the conclusions from the pH-stat studies and strongly support a channel mechanism.

4.3. 23Na NMR

In order to further validate the channel activity of the 21-mer peptide and to screen more analogues, a dynamic NMR method has been used. This method allows a good evaluation of sodium channel transport activity without any driving force. Based on the differentiation of Na+ inside and outside phospholipid vesicles by the use of Dy3+ as shift reagent, this technique provides an exchange rate of Na+ ions through model membranes and has been successfully applied to gramicidin and artificial ion channels [53,61,62]. Studies have demonstrated that the transport activity of the 21-mer peptide, even if it corresponds to 3% of that of gramicidin D, is 30 times higher than that of an analogue bearing smaller rings (18-crown-6) and more than 600 times higher than that of peptides with even smaller rings (14-crown-4 and 13-crown-3) [48]. This strongly supports the monomolecular channel mechanism of the 21-mer
peptide and the hypothesis that Na\(^+\) ions travel through the crown ring channel instead of through a pore formed by the aggregation of peptides, which is in agreement with CD and patch clamp studies. Furthermore, addition of a non-ionic hydrophilic head group at the N-terminal of the 21-mer peptide (peptide D) resulted in an increase by 2.7 times of the Na\(^+\) transport activity, while the transport is only slightly improved for C-polar terminal 21-mer peptide (peptide E) and N,C-polar termini 21-mer peptide (peptide F) [63].

These results suggest that the addition of a polar group at the N-terminal position increases the stability of the peptide in the membrane without decreasing its incorporation. Furthermore, it demonstrates that the transport activity can be enhanced with different peptide extremities and opens the way to the incorporation of molecular recognition elements for selective targeting. Finally, the results obtained with the 14-mer peptide indicate a similar transport activity than for the 21-mer peptide, even if the length of the peptide is shorter than the thickness of the bilayer. This is most likely due to the fact that the lysis of vesicles produces the same signal as transport. It is therefore very difficult to discriminate the two phenomena by this NMR technique.

### 4.4. Fluorescence studies

Fluorescence assays have also been performed to verify that the activity observed by \(^{23}\)Na NMR for the 21-mer peptide is not due to the lysis of vesicles and to demonstrate the lytic ability of the 14-mer peptide. The vesicle lysis experiment is based on the increase of the self-quenched fluorescence of calcine upon lysis [47,64]. Fig. 3 shows the calcine release profile induced by the addition of the 14-mer and the 21-mer peptides to lipid vesicles. These results indicate that the 21-mer peptide induces very little lysis, which is also the case for the N-polar terminal 21-mer peptide (peptide D), C-polar terminal 21-mer peptide (peptide E) and N,C-polar termini 21-mer peptide (peptide F) bearing one or two polar headgroups (data not shown) [48,63]. These results confirm that the activity observed in the \(^{23}\)Na NMR assay reflects the transport ability of the 21-mer artificial channel. On the other hand, the 14-mer peptide clearly induces a rapid and significant release of calcine. This particularity demonstrates the ability of the 14-mer peptide to act as a lytic agent as predicted.

### 5. Effects of the 14-mer and 21-mer peptides on model membranes

This section will be devoted to the study of the effect of the 14-mer and 21-mer peptides on lipid bilayers by a combination of \(^{31}\)P and \(^{2}\)H solid-state NMR spectroscopy. These two techniques allow the study of both the polar and the hydrophobic regions of the lipid bilayer. Both peptides have been investigated with fully hydrated zwitterionic multilamellar vesicles composed of dimyristoylphosphatidylcholine (DMPC) at lipid-to-peptide molar ratios of 60:1 and 20:1, and with fully hydrated mechanically oriented bilayers of dioleoylphosphatidylcholine (D LPC) and DMPC at a lipid-to-peptide molar ratio of 60:1.

#### 5.1. \(^{31}\)P NMR spectroscopy

Phosphorus-31 has a spin-1/2 and a 100% natural abundance, and, due to its good sensitivity, is extremely useful to investigate the structure and dynamics of the polar headgroup of phospholipids, which are the main constituent of biological membranes [65]. In particular, it is possible to obtain static \(^{31}\)P NMR spectra which are dominated by the chemical shift anisotropy (CSA), to use the magic-angle spinning (MAS) technique that averages the CSA to its isotropic value, and finally to use samples macroscopically oriented in the magnetic field. The general conclusions that will be presented in this section for the 14-mer and 21-mer peptides in interaction with lipids refer to the shape of the vesicles, the dynamics and/or orientation of the polar headgroup upon peptide binding to unoriented vesicles, and also on the perturbing effect of the peptides on membrane orientation and the influence of acyl chain length on the interaction between peptides and mechanically aligned bilayers of DLPC and DMPC.

The \(^{31}\)P NMR spectra of the DMPC vesicles revealed that the 14-mer and the 21-mer peptides increase the dynamics and/or induce changes in the polar headgroup orientation since a decrease of the CSA has been observed. The shape of the DMPC vesicles in the presence of the 14-mer and the 21-mer peptides also demonstrate a greater 0\(^\circ\) orientation of the lipidic molecules, i.e., with the lipid long axis preferentially oriented parallel to the magnetic field direction upon peptide binding. A partial 90\(^\circ\) orientation is observed for the pure system, i.e., an ellipsoidal vesicular shape in which the lipid long axis is oriented preferentially perpendicular to the magnetic field direction [66,67]. The greater 0\(^\circ\) orientation is reflected on the \(^{31}\)P NMR spectra by an increase of the spectrum shoulder at approximately 30 ppm. The change in the DMPC vesicular shape could be explained by a change in the elastic properties of the vesicles or by a modification of the magnetic susceptibility of the lipid molecules upon peptide binding [68–70].

![Fig. 3. Calcein leakage induced by the addition of the 14-mer and the 21-mer peptides (5 mM) to phosphatidylglycerol/phosphatidylcholine (1:1) vesicles in HEPES 100 mM, NaCl 170 mM, EDTA 5 mM at pH 7.4. Vesicles were lysed with Triton X-100 at 400 s. Adapted from reference [47] and reproduced with permissions.](image)
A decrease in the CSA is in good agreement with changes in the dynamics and/or orientation of the lipid polar headgroup. Such perturbation could be explained by the amphipathic nature of the peptides. When adsorbed at the bilayer surface, the crown ethers are facing the aqueous phase while the hydrophobic contour of the helix is facing the hydrophobic core of the bilayer. This interfacial interaction could result in a change in the dynamics and/or orientation of the polar headgroup. Similar results have been reported by Bonev et al. who have studied the interaction between DMPC and sphingomyelin (10% mol) membranes and equinatoxin II [71]. Huster et al. have also studied the colicin Ia channel in interaction with POPC/POPG vesicles and they concluded that colicin Ia interacts with the lipid bilayer since a smaller 31P CSA has been observed [72]. Several other groups have also performed 31P NMR experiments on antimicrobial and human peptides [73–76]. They all attributed a decrease in the CSA to a perturbation of the lipid headgroup packing.

We have also used 31P NMR to investigate the interaction between the 14-mer and 21-mer peptides with lipid bilayers oriented between glass plates [77–79] prior to the determination of their membrane topology by 15N NMR spectroscopy. Two different types of lipids have been used, namely DLPC and DMPC that possess acyl chains of 12 and 14 carbon atoms, respectively. The choice of lipids has been made by considering the hydrophobic match/mismatch between the bilayers and peptides. Assuming that the 14-mer and 21-mer peptides adopt a perfect α-helical structure, their length would be 21.0 Å and 31.5 Å, while the hydrophobic thickness of DLPC and DMPC is estimated at 20.0 Å and 23.0 Å, respectively [80,81]. The 31P NMR oriented spectra of pure DLPC and DMPC bilayers (Fig. 4, solid lines) show sharp resonances at 31.2 ppm and 30.7 ppm, characteristics of lipid bilayers oriented with their normal parallel to the magnetic field direction.

In DLPC bilayers, the 14-mer peptide (Fig. 4A, top trace) induces a broadening of 0.4 ppm and a shift to lower chemical shift (upfield shift) of 2.0 ppm, while the 21-mer peptide (Fig. 4A, bottom trace) induces a narrowing of the resonance by 0.5 ppm and an upfield shift of 1.0 ppm. In DMPC bilayers, there is a narrowing (broadening) of the resonance by 0.2 (0.2 ppm) with the 14-mer (21-mer) peptide (Fig. 4B, top trace (bottom trace)). Since opposite effects are observed for the 14-mer and 21-mer peptides in DLPC and DMPC, and that the length of the 14-mer peptide fits the hydrophobic length of DLPC bilayers while the length of the 21-mer peptide better fits the hydrophobic length of DMPC bilayers, we can suppose that the hydrophobic match/mismatch affects the peptide/lipid interactions. Based on these results, we can expect a transmembrane (in-plane) orientation of the 14-mer peptide in DLPC (DMPC) bilayers, while the 21-mer peptide is most susceptible to be oriented in an in-plane (transmembrane) orientation in DLPC (DMPC) bilayers.

We cannot however rule out the possibility that the 21-mer peptide could be tilted in DLPC bilayers to compensate for hydrophobic mismatch instead of having an in-plane orientation. A similar case has already been reported by Park et al. who have studied the variation in the tilt angle of the transmembrane helix of Vpu peptide depending on the bilayer thickness [82]. They reported that the tilt angle is changed from 27° in 14:0-PC/PG to 51° in 10:0 PC/10:0 PG lipid bilayers.

5.2. 2H NMR spectroscopy

2H NMR spectroscopy is a very powerful technique to study the membrane hydrophobic core by replacing the acyl chain protons by deuterons. Deuterium is a spin-1 nucleus with a quadrupole moment that interacts with the electric field gradient at the nucleus, giving rise to the quadrupolar interaction. Two spin transitions are possible and a doublet of resonances is observed on a 2H NMR spectrum, separated by the quadrupolar splitting ΔνQ. For a system with axially symmetric motions, the quadrupolar splitting is given by:

\[ ΔνQ = \frac{3}{4}(e^2qQ/h)(3\cos^2θ - 1)S_{CD} \]  

where \( (e^2qQ/h) \) is the quadrupole coupling constant (~170 kHz for aliphatic C–D) [83], \( θ \) is the angle between the bilayer normal and the external magnetic field \( B_0 \), and \( S_{CD} \) is the order parameter of a deuterium bond vector. As described extensively, this order parameter is the product of several contributions,

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![Chemical shift, ppm](image_url)
including intramolecular motions such as trans-gauche isomerizations, and anisotropic reorientation of the whole phospholipid molecules. Therefore, it is possible to determine variations in lipid chain order by monitoring changes in $\Delta \nu_Q$ values \[84\].

The $^2$H NMR spectra of the DMPC vesicles in the presence of the 14-mer peptide show an increase in the quadrupolar splitting $\Delta \nu_Q$ at the plateau region while there is no change in $\Delta \nu_Q$ upon the 21-mer peptide binding \[66\]. The increase in $\Delta \nu_Q$ reflects the stabilization on the lipid acyl chains in the presence of the 14-mer peptide. These results suggest that the peptide is located at the surface of the bilayer. Dufourc et al. have observed a similar behavior with melittin \[85,86\]. They associated their results to a location of melittin at the surface of the bilayer, capping the lipid headgroups and leading to a greater chain packing. A deeper insertion of melittin in the bilayer results in a disordering effect of the hydrophobic core. This situation could be explained by the presence of a hydrophobic mismatch between melittin and the lipid bilayer. In our case, however, no change is observed in the packing of the DMPC acyl chains in the presence of the 21-mer peptide. This could be explained by the fact that the 21-mer peptide and the DMPC bilayers do not present a significant hydrophobic mismatch, which would support a transmembrane orientation of the 21-mer peptide in the bilayer. However, a location of the peptide at the bilayer surface with no direct effect on the acyl chain ordering is also conceivable. De Planque et al. concluded that a hydrophobic match between the bilayer and the peptide gramicidin A results in a non-perturbed bilayer since no adjustment has to be done to compensate for hydrophobic mismatch \[81,87\]. Belohorovcova et al. also reported that a membrane spanning peptide has little effect on lipid chain order if its hydrophobic length closely matches the lipid hydrophobic thickness \[88\]. Hence, the 14-mer peptide seems to be located at the surface of the bilayer, while the topology of the 21-mer peptide has not yet been precisely determined. Further experiments such as $^{15}$N NMR experiments in oriented bilayers are currently underway with both peptides to conclude on their membrane topologies.

6. Proposed mechanisms of action

The aim of the present work was to shed light on the interactions between synthetic amphipathic peptides and model membranes to better understand the mechanisms of action of antimicrobial peptides. First, circular dichroism has been used to confirm the helical conformation of the 14-mer and 21-mer peptides both in solution and bound to lipid bilayers. Under such a helical conformation, crown ethers are sequestered on one side of the $\alpha$-helix and confer an amphipathic character to the peptides. Secondly, pH-stat and single-channel measurements, and $^{23}$Na NMR spectroscopy have shown that the 21-mer peptide acts as an artificial ion channel by promoting the transport of ions across the lipid bilayer. In addition, calcein leakage experiments by fluorescence spectroscopy, $^{23}$Na NMR spectroscopy and lysis of erythrocytes \[47\] by visible spectroscopy have all confirmed the permeabilization activity of the 14-mer peptide and the non-lytic activity of the 21-mer peptide. Since ion transport studies indicated that both peptides interact in a different manner with lipid membranes, $^{31}$P and $^2$H solid-state NMR studies have been done on multilamellar vesicles and oriented membranes to gain information on the interaction of the peptides with lipid bilayers. These NMR experiments have allowed us to probe the effects of the 14-mer and 21-mer peptides at two different regions of the bilayer, namely the polar headgroup and the hydrophobic core. All these experiments have permitted us to suggest models of interaction as depicted in Fig. 5.

The results strongly suggest that the 14-mer peptide is located at the surface of the DMPC bilayer in an in-plane orientation that minimizes the hydrophobic mismatch between the peptide and the bilayer and it interacts with the polar headgroup of the lipids. The perturbing effect of the polar headgroup reflected on the $^{31}$P NMR spectra as well as its perturbing effect on the membrane permeabilization could be explained by its inverse-cone shape. According to Epand et al., this peptide belongs to the structural class A in which the region occupied by the hydrophilic part on the amphipathic helix is larger than the hydrophobic region, and this shape is recognized to induce positive curvature in the bilayer \[33\]. Such effect has also been reported by Ramamoorthy et al. who have studied the disruption of the lipid bilayer induced by an analogue of magainin, MSI-78 \[89\]. The induction of a positive curvature by the 14-mer peptide is also supported by a net decrease of calcein leakage of vesicles made of PC and PE lipids \[47\]. The PE lipids possess a cone shape that counter-balances the inverse-cone shape of the 14-mer peptide, and then inhibits the positive curvature imposed on the lipid bilayer. Single-channel measurements and $^{23}$Na and solid-state NMR experiments both suggest a partial transmembrane orientation of the 21-mer peptide in a DMPC lipid bilayer. Since there is no significant hydrophobic mismatch between the peptide and the DMPC bilayer, a transmembrane topology of the 21-mer peptide

![Fig. 5. Proposed models for the binding of the 14-mer (left) and 21-mer (right) peptides to DMPC bilayers. Those cartoons represent a static caption of the lipid/peptide interaction and are not drawn on scale. The 14-mer peptide is inserted in the hydrophobic core of the bilayer and adopts an in-plane orientation with the leucine hydrophobic side chains interdigitating the hydrocarbon lipid chains. In DMPC bilayers, the 21-mer peptide is in equilibrium between in-plane and transmembrane orientations that are consistent with previous single-channel conductivity and ATR studies (see text for details).](image-url)
could account for its single-channel activity. Previous ATR studies have also suggested that the 21-mer peptide could be in equilibrium between in-plane and transmembrane orientations [54], as depicted in Fig. 5.

7. Conclusions

The present study has demonstrated the utility of the design and synthesis of artificial amphipathic peptides to better understand the mechanisms of action of antimicrobial peptides. A better knowledge of the interactions that support these modes of action opens the way to novel classes of antibiotics with different biological activities that defy most known mechanisms of resistance. The study has also shown the potential of various biophysical and spectroscopic techniques to bring complementary information that can be used to shed light on the mechanisms of action of synthetic amphipathic peptides.

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