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Interaction of the antibiotic peptide nisin with anionic membranes in different phase-states: a vibrational study

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Abstract

Interactions between the antibiotic peptide nisin and multilamellar vesicles of phosphoglycerol lipids in different phase-states were studied using vibrational spectroscopy. The infrared amide I' band of nisin, both in solution and in the membrane-bound state, was analyzed in the temperature range comprised between 20 and 60°C in order to study its conformational behavior. Nisin presented mainly unordered and β-turns conformations. Their relative populations varied according to the environment and as the temperature increased: β turns were more favored in the membrane-bound state than in solution, but at higher temperatures the disordered conformation was dominant in both states. Spectral changes of specific infrared bands belonging to the hydrocarbon and polar moieties of lipids were also analyzed to evaluate the perturbation of the lipid membrane order. Nisin interactions with the membrane polar region induced a high restriction to water incorporation, promoting a small increase in the temperature of the lipid phase transition. Raman spectra of nisin/phosphoglycerol systems at ambient temperature were also analyzed. They revealed that the peptide incorporation to a membrane in the fluid phase

caused drastic structural modifications in the hydrophobic region of the bilayer. Although nisin may be able to disrupt the hydrophobic portion of the bilayer in the gel phase, the most of the peptide molecule remained at the membrane surface interacting with the polar headgroups. This work provides evidence of a differential effect of nisin on anionic membranes, depending on the phase-state of the lipid.

Keywords

NISIN, VIBRATIONAL SPECTROSCOPY, ANIONIC MEMBRANES

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1. Introduction

Phospholipid bilayers are the basic structure of lipid membranes. Their structural and dynamic properties have been extensively studied to understand the large number of biochemical processes that occurs within cell membrane systems [1-3]. Among them, we are interested in studying the interactions with the lipid membrane and the functions of some membrane-active compounds such as antimicrobial peptides. Nisin, well known because of its antibiotic activity, is a small peptide, ribosomally synthesized by different strains of *Lactococcus lacti*s [4], carrying a net positive charge (+5) at neutral pH. It is made up of 34 amino acids (Figure 1), some of which are rare and naturally originated in post-translational modifications of serine and threonine, the dehydroalanine (Dha) and dehydroaminobutyric acid (Dhb), respectively. Another structural characteristic of this peptide is the presence of five intramolecular thioether rings of Lanthionine (Ring A, in Figure 1) and Methyllanthionine (Rings B to E) that confer a singular stability to the polypeptide chain [5], and which resulted from the reaction between the dehydrated Dha or Dhb amino acids and the SH group of neighboring cysteine residues [6,7]. Due to these structural features, nisin belongs to a group of bacteriocins known as lantibiotics [7,8]. Although the structure of nisin cannot be described in terms of regular secondary-structure elements due to the 65% of the residues are incorporated in the ring systems, some well-defined structural features have been observed by 1H NMR for the peptide in water [9]: the four-residue rings B, D and E all show a β-turn structure, while in ring A, a γ-turn structure is adopted around Dha5. Ring C has the least defined structure, and together with the region comprising the residues 29-34 that presents an extended conformation, shows high flexibility.

Nisin has been used in industry for decades as a food preservative because of its innocuousness in human beings [10] and its antibiotic activity against a broad spectrum of Gram (+) bacteria [11-13]. Microbiological studies have probed that nisin inhibits bacterial growth and

causes cell lysis [12,14,15], being the cytoplasmic membrane its primary target of action where it forms discrete pores that disrupt the membrane integrity [16]

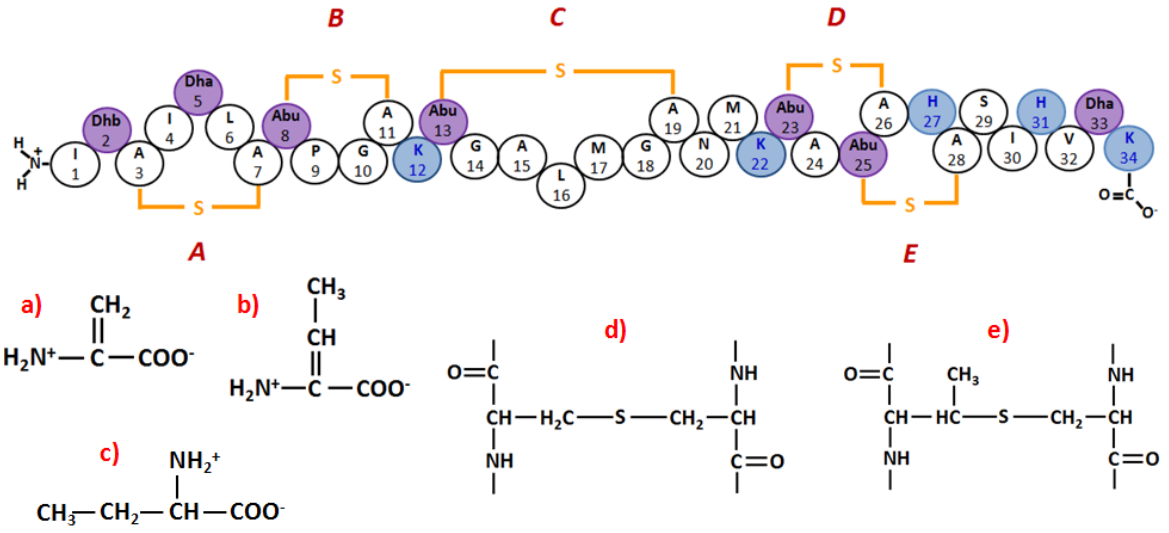

Figure 1: Nisin A primary structure. Amino acids plotted in blue are positively charged (+) at pH=7. Post-translational modified amino acids are shown in purple. Rings are named from A to E. Structure of unusual amino acids: a) Dehydroalanine (Dha), b) dehydroaminobutyric acid (Dhb) and c) 2-aminobutyric acid (Abu). d) Lanthionine(A-S-A) and e) Methyllanthionine (Abu-S-A)

In order to achieve a better understanding of the mechanisms involved in the antibiotic activity of nisin, several investigations were performed in both bacterial strains and model membrane systems. On one hand, it was demonstrated that the main mechanism of nisin action is associated to the presence of the Lipid II (LII) molecule in the membrane, the bactoprenol-bound precursor of the bacterial cell wall, which is used by the peptide as a docking molecule for the subsequent pore formation [17,18]; on the other hand, strong evidence that the amphiphilic properties of nisin allow it to interact directly with the lipidic portion of the membrane have been

obtained, based on the short-lived pores that the peptide forms in natural or synthetic lipid bilayers [9].

The mechanism of pore formation by nisin in model membranes has been extensively addressed. Although a broad agreement was reached regarding the primary stage of action mechanisms, some discrepancies arose about the subsequent membrane disruption process. Thus, the observed high affinity for anionic membranes indicated that the nisin binding is mainly driven by electrostatic interactions; since the nisin C-terminal domain contains the major part of the positive charge of the molecule, it was proposed that this domain is responsible for the initial binding of nisin to the membrane surface [9,19-23]. However, some authors concluded that the electrostatically surface-bound nisin perturbs the lipid bilayer structure without affecting the global permeability of the anionic membrane, indicating that nisin behaves as an extrinsic peptide [19,20]. On the contrary, other authors have confirmed the ability of nisin to insert into the hydrophobic lipid region and disrupt the membrane integrity, proposing different models for pore formation. Binding studies showed that wild type nisin, as well mutants containing an unique Trp residue at three different positions, experience aggregation at the surface membrane of anionic phospholipids [21,22], which would confirm the early suggestion about the mode of action of nisin based on the barrel-stave model [24]; this model involves the initial accumulation of the peptide at the membrane surface through ionic interactions and the subsequent pore formation. Almost simultaneously, it was proposed that the pore is formed by translocation of the C-terminal region of the nisin molecule across the membrane. In that case, after the electrostatic binding of the nisin to the bilayer through its C-terminus, the insertion of the N-terminal part of the molecule into the outer leaflet of the membrane follows, so that finally the entire peptide can be translocated across the membrane [23]. A wedge model has also been proposed for pore formation by nisin. Here, the positively charged C-terminus, together with the bound lipids, enter into the membrane forming a

wedge-like pore composed of multiple nisin molecules [25,26]. Most of the mentioned information concerning the effect of nisin on the membrane integrity has been acquired by fluorescence techniques and NMR spectroscopy.

On the other hand, in order to contribute to the elucidation of the peptide structure-function relationship, 1H NMR studies of nisin structure in aqueous solution and in membrane-mimicking environments showed conformational changes upon lipid binding, mainly affecting the ring A and the segment 21-24, which adopts a β-turn conformation upon micelle interaction [9]. Those results were later confirmed by infrared spectroscopy of the peptide in the free and the membrane-bound states; the behavior of the amide I band of nisin was indicative of increment in the β-turn content [20]. However, binding analysis of nisin to anionic membranes in different lipid phases, also included in the last report, indicated that the peptide interacts mainly at the membrane interface level and the contact with the hydrophobic core of the bilayer is limited, regardless of the lipid phase [20]. Such results are difficult to correlate with any of the model for pore formation above mentioned, as well with recent studies that show that both the liquid-crystalline (L_{α}) and the gel ($L_{\beta'}$) lipid phases play important roles in lipid interaction with antimicrobial peptides [27,28].

To the best of our knowledge, no studies based on vibrational behavior of the lipid bilayer upon nisin interaction have been reported yet. Vibrational spectroscopy is especially suitable to provide additional information on the mechanism of pore formation and permeabilization of the membrane by nisin. Fourier transform infrared spectroscopy (FTIR) and Raman microspectroscopy (RMS), can produce valuable and sensitive information about structural aspects such as the membrane packing, the proportion of confinement in the hydrocarbon chains, the degree of hydration of the lipid head, the groups and the conformation of peptides, as well as the changes in all of them induced by specific interactions [29-33].

Recently, we focused on the structural characterization, at a molecular level, of the effect exerted by nisin in model lipid systems that mimic the bacterial membrane by using vibrational spectroscopy. Based on evidences of a dual behavior of the peptide, depending on whether the membrane contains or not LII, it is necessary to have a detailed knowledge of the specific interactions that occur between the different components of the studied systems. In this context, the spectral behavior of multilayer vesicles in liquid-crystalline and gel phases formed by pure PG lipids and by PG/LII mixtures have been already studied by Raman microspectroscopy. The differences shown by specific spectral markers of the lipid structure were associated with perturbations on the bilayer as response to the LII incorporation [34].

In the present work, we use vibrational spectroscopy to study systems containing nisin free in solution and bound to lipid membranes in order to complete the current knowledge about the pore formation process and to provide a better understanding of how lipids and peptide are mutually influenced in dynamics and conformation. We studied the influence of nisin on the structure of lipid membranes of dilauroylphosphatidylglycerol (DLPG) and dipalmitoylphosphatidylglycerol (DPPG) in gel or liquid-crystalline phases. We also measured the conformational changes of the peptide at different temperatures. The selection of such lipid systems was based on: i) PG lipids are the most abundant components of bacterial membranes and the high binding affinity of nisin for anionic lipid membranes have been already demonstrated [9,19-21,23]; ii) The main transition temperatures (T_m) for DPPG and DLPG lipids are 41 °C and -5 °C, respectively, making it possible to study systems in the ordered phase ($L_{\beta'}$) and in the fluid phase (L_α) by collecting Raman spectra solely at ambient temperature (20 °C) [34,35]. Thermotropic studies of free and membrane-bound nisin, performed by FTIR measurements within the 20-60°C temperature range, were also included. Although the L_α phase is the most biologically relevant in biological systems, the study of membrane properties below the

temperature of chain melting transition has gained importance since it was demonstrated that some antimicrobial peptides can shift the T_m of bacterial model membranes [27].

Our results indicate that the degree of perturbation by nisin binding at both the polar headgroup level and at the hydrophobic region of the membrane is strongly dependent on the lipid phase. In turn, conformational changes in the peptide molecule are dominated by the lipid environment, which counteracts, at least partially, the temperature effect. All these results, in conjunction with those previously obtained from the Raman analysis of the DLPG/LII and DPPG/LII systems [34], will be of relevance to further analyses and interpretation of spectra of the ternary DLPG/LII/nisin and DPPG/LII/nisin complexes, which are expected to contribute significantly to the understanding of the molecular bases of nisin activity.

2. Material and Methods

2.1. Sample preparation

DPPG and DLPG were obtained from Avanti Polar Lipids and used without further purification. Nisin A (CHRISIN®) was isolated and purified as described previously [36]. PG lipids were dissolved in chloroform:methanol (2:1) and dried as a thin film under a nitrogen stream. For FTIR measurements, samples were prepared in deuterium oxide (^2H_2O) to avoid the water interference in relevant spectral regions; H_2O absorbs strongly at approximately 1640 cm^{-1}, making it difficult the deconvolution and peak fitting procedures of peptides amide I bands [37]. DPPG films were re-suspended by vortexing in ^2H_2O solution containing 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), 50 μM ethylenediaminetetraacetic acid (EDTA) and 50 mM NaCl. Nisin was separately dissolved in the same deuterated buffer at ambient temperature (20°C) and then added to DPPG vesicles to give a mixture of 5:1 (PG:nisin) molar ratio. Based on a

published binding study of nisin to phosphatidylglycerol multilamellar vesicles, it is expected that most of the nisin is bound to the lipid membranes at this molar ratio [20]. For Raman measurements, nisin, DPPG and DPPG/nisin mixture samples were prepared in the same conditions as mentioned but by replacing ^2H_2O solution by tridistilled water. In addition, DLPG and nisin/DLPG mixture were also prepared at ambient temperature. The final concentration of PG in all samples was 20mM.

2.2. Raman microspectroscopy measurements

Raman spectra between 3500 and 50 cm^{-1} were acquired in a DXR Raman Microscope (Thermo Fisher Scientific). Data were collected using a diode-pump, solid state laser of 532 nm at 10 mW of power (4 cm^{-1} spectral resolution). A confocal aperture slit of 50 μm was used. A drop (~ 20μL) of each solution sample was placed on a gold-coated sample slide and a 10× objective was used. In order to achieve a sufficient signal-to noise ratio, spectra were collected from each sample by accumulating 300 scans with an exposure time of 4s each one. A total number of three spectra was collected for each sample. All Raman experiments were carried out at ambient temperature (20°C) to ensure that multilayer vesicles of DPPG lipids in pure state and in the nisin/DPPG mixture were in the gel (L_{β}') phase, and that the multilayer vesicles of DLPG lipids in pure state and in the nisin/DLPG mixture were in the liquid-crystalline state (L_{α} phase).

2.3. FTIR spectroscopy measurements

FTIR spectra between 4000 and 1000 cm^{-1} were acquired in a Nicolet Nexus spectrometer using a thermostated demountable cell for liquid samples with CaF_2 windows and a 76 μm teflon spacer. The spectrometer was flushed with dry air to reduce water vapour distortions in the spectra. 32 scans were collected both for the background and the sample at a nominal resolution

of 2 cm^{-1}. The temperature in the cell was controlled with a circulating water bath. The temperature setting in the bath was increased in steps of about 2 °C, from 20°C to 60°C. This temperature range allows the study of the gel and liquid-crystalline lipid phases by using only the nisin/DPPG mixture. The actual temperature in the sample, measured with a thermocouple inserted in the cell, increased continuously with a scan rate of ~ 0.5°C/min.The acquisition of a spectrum (32 scans) at a given nominal temperature spanned 1 min. Thus, the change in the temperature was about 2 °C between consecutive spectra. FTIR spectra were acquired from two different samples of each system to check reproducibility of data.

2.4. Data analysis

All spectral data were processed with the OMNIC 8.3.103 Software suite (Thermo Fisher Scientific Inc.) The spectral contribution of the ^2H_2O solution was eliminated from the FTIR spectra of nisin and nisin/DPPG by subtracting the absorbance of the ^2H_2O solution acquired at the corresponding temperature; this procedure yields the baseline correction in the region of the Amide I band [38]. Each Raman spectrum was baseline corrected in the entire spectral range, while no solvent subtraction was applied to these spectra because of the negligible spectral contribution of water. Due to the low variability observed among the Raman spectra independently collected from a same sample, and in order to increase the signal-to noise ratio, a single, average spectrum was later generated for each system by taking the arithmetic mean of the corresponding spectra [34]. The location and relative intensities of the overlapping components in specific spectral regions were mathematically decomposed using the GRAMS/AI 8.0 Spectroscopy Software (Thermo Electron Corporation). Band components were considered as Voigt functions that combine Gaussian and Lorentzian characters and allow each portion to have different line widths [39]. First, the number of individual peaks was estimated from the second

derivative of the spectra. Then, the fitting was performed iteratively allowing parameters to float without limits until convergence to a local minimum was attained [34,35,40]. The quality of the fitting was evaluated by overlapping the reconstituted overall curve on the original spectrum and by matching the position of each band component with the corresponding location in the second derivative spectrum. In addition, wavenumbers of the band components were compared to previous results. Thus, wavenumbers of all component bands of the FTIR amide I´ of nisin at 20°C were in good agreement with those already reported for nisin in $^{2}H_2O$ [20]. Similarly, Raman component bands, comprised in the 1150 - 1020 cm^{-1} spectral region of the DLPG and DPPG multilayer vesicles, were all in close concordance with those previously reported by our group for the same lipid systems in pure state [34,35].

3. Results and Discussion

3.1. FTIR assays at different temperature. Nisin structure in aqueous solution and bound to micelles and phospholipid bilayers has been previously studied by 1H NMR [9,41] and IR spectroscopy [20]. Those works showed that nisin has a high content of β-turns and unordered conformations and that the secondary structure of nisin is sensitive to the environment, despite the molecular rigidity conferred by the five lanthionine rings.

We have further studied the structural changes of nisin in solution and bound to multilamellar bilayers of DPPG as a function of temperature using FTIR spectroscopy. Figure 2 shows the spectral region corresponding to the amide I´ band (1700 - 1560 cm^{-1}) of nisin in $^{2}H_2O$ solution and in DPPG membranes within the temperature range 20 to 60°C. The amide I´ band profile of nisin in solution at 20°C showed a maximum at 1645 cm^{-1} and a shoulder at 1632 cm^{-1},

which were assigned to unordered and β-turns conformations, respectively [20,42]. At approximately 34°C, the feature at 1645 cm^{-1} started a constant shift to higher wavenumbers with the increase in temperature (up to 1651 cm^{-1} at 60°C), while increasing its relative absorbance to the shoulder at 1632 cm^{-1}, as shown in the inset in Figure 2. The continuous increment in the intensity ratio suggested that the transition was not between two well-defined conformations separated by a large energy gap. Instead, nisin shifted between conformations that slightly changed their populations as the temperature was increased. A previous structural analysis of nisin in aqueous solution demonstrated the absence of a well-defined structure in ring C as well as certain flexibility in both the N-terminus and C-terminus and in the residues Asn20 and Met21 [9]. Then, one might expect that these regions show a greater sensitivity to temperature than the rest of the well-structured ring system that characterizes the molecule. Upon nisin incorporation to DPPG vesicles, at 20°C, the amide I´ band profile experienced a slight narrowing and the shoulder at 1632 cm^{-1} was better defined than in solution. More meaningful, however, was its thermotropic behavior: the feature attributed to unordered conformations shifted from 1648 to 1652 cm^{-1} within the temperature range 34 to 44°C and remained at the same wavenumber despite further increases in temperature. By plotting the absorbance ratio A_{max}/A_{1632} as a function of temperature (see the inset in Figure 2) two different situations were observed: i) above 34°C the absorbance ratio decreased indicating an increment in the proportion of β-turns; ii) when the main lipid phase transition has occurred (above 44°C), the absorbance ratio raised slightly, suggesting increment in unordered conformations as a temperature effect.

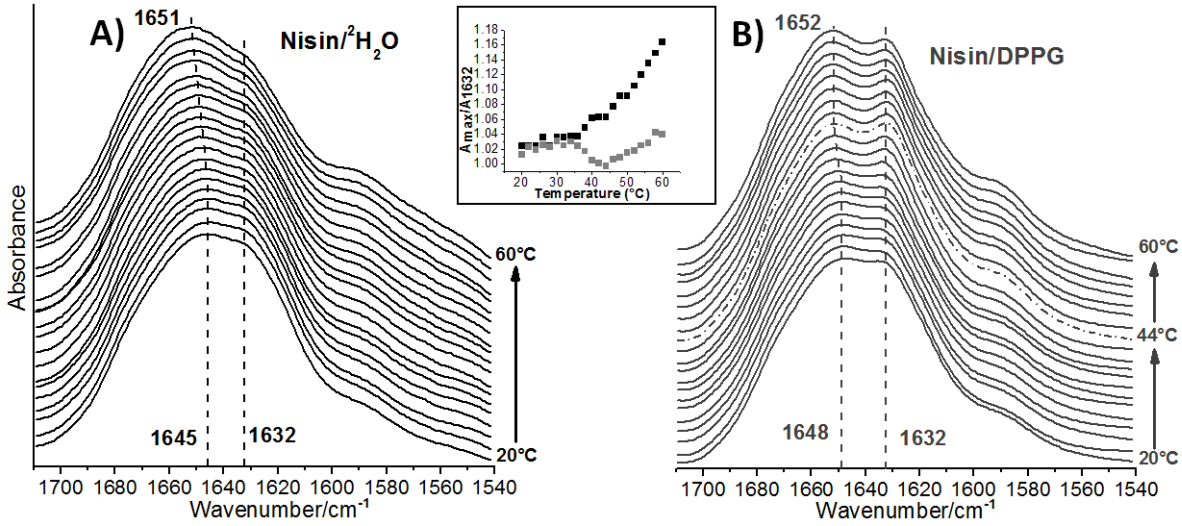

Figure 2: IR amide I' band of nisin in the temperature range going from 20°C to 60°C. A) nisin in ^2H_2O solution, B) nisin in DPPG membranes. Spectra were recorded every 2°C. Inset: Temperature dependence of absorbance ratio between the band maximum and the shoulder at 1632 cm^{-1}.Black and gray symbols corresponding to pure nisin in solution and nisin/DPPG system, respectively.

Although weak, these differences in the shape of the amide I´ band evidenced the sensitivity of the peptide structure to both temperature and environment, and could be better appreciated by spectral decomposition. Figure 3 shows the components of the amide I´ band of nisin in solution and bound to DPPG vesicles at different, selected temperatures. In all the cases, four individual components were observed, whose positions were in close agreement with those previously reported for the peptide in similar environments [20]. The component at 1646 - 1654 cm^{-1} was assigned to unordered structures [20], while the components at 1624 - 1629 cm^{-1} and 1668 - 1673 cm^{-1} were assigned to β-turn conformations according to Hóllosi et al. [42]: the former was attributed to amide C=O groups involved in the strong 1←4 intramolecular hydrogen bond

that stabilizes the β-turns, while that at higher wavenumber was assigned to the "free" carbonyls (not involved in the intramolecular H bond) that integrate the β-turns. The weak component observed at 1588 - 1593 cm^{-1} was assigned to the lateral chain vibrations of histidine and/or to the asymmetric stretching mode of the terminal COO^- group [20]. In the spectrum of nisin in ^2H_2O at 60°C (Figure 3 B), the component attributed to unordered conformations appeared enhanced in approximately 25% with respect to the β-turn components, upon comparison with the relative absorbance of the component bands at 20°C (Figure 3 A), indicating that disruption of some β-turns was induced by the increase of temperature. The shifts of the components toward higher wavenumber as temperature increased can be due to the weakening of the intermolecular hydrogen bonds [43]. However, it has been demonstrated that the upshifts of the amide I band of peptides in ^2H_2O can not be entirely due to hydrogen bonding, but rather arise from the temperature dependence of the solvent dielectric properties as well [44].

Fourier self deconvolution and band fitting of amide I´ band of the nisin/DPPG system at 20°C (Figure 3 C) revealed the components associated to unordered and β-turn conformations shifted towards higher wavenumbers (1649 and 1628 cm^{-1}, respectively) with respect to the free nisin in ^2H_2O at the same temperature, but no relevant differences in the relative absorbance were observed, suggesting that the peptide did not experienced structural changes when interacted with the membrane in the gel lipid phase. Two main spectral differences were observed for the complex system at 44 °C (Figure 3 D): i) the component at 1628 cm^{-1} became stronger than that corresponding to unordered conformations, shifted to 1654 cm^{-1}; ii) the contribution of the component assigned to the intermolecular hydrogen-bonded carbonyls decreased significantly (1673 cm^{-1}). These changes strongly suggest that the peptide was able to penetrate into the hydrophobic region of the membrane, which is in a phase more fluid at that a temperature, such that the changes in the secondary structure of nisin may be due to a more hydrophobic

environment. A previous study by 1H NMR of nisin incorporated to micellar systems reported a change in the secondary structure of the peptide; specifically, the segment Met21-Ala24 forms a β-turn in the bound state, while this structural feature is no observed for free nisin [9]. In the nisin/DPPG system at 60°C, the component at 1653 cm^{-1} was dominant (Figure 3 E). This structural change was interpreted as an effect of temperature and not of the environment.

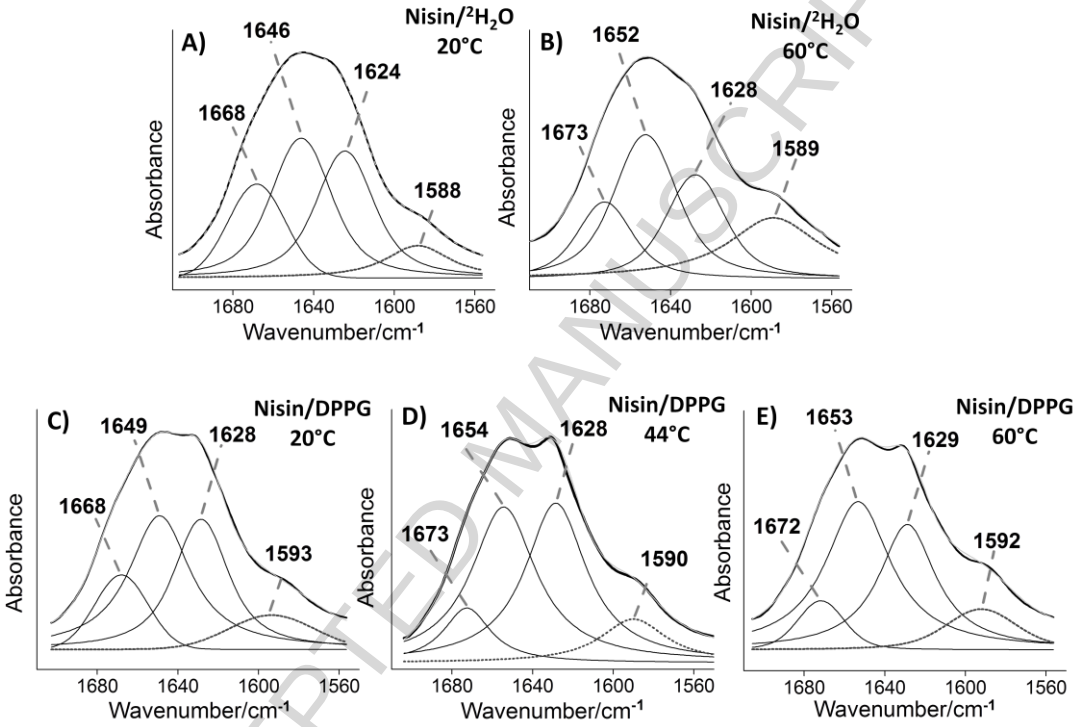

Figure 3: Spectral decomposition of the IR amide I' band of nisin. A) and B) peptide in solution at 20 and 60 °C, respectively. C), D), and E) nisin/DPPG system at 20, 44, and 60°C, respectively.

The influence of nisin on the lipid phase transition was also studied by IR spectroscopy. Two specific lipid vibrations were evaluated: the C=O stretching mode (υC=O) of carbonyl groups located in the interfacial region of the membrane, appearing as a strong band at ~ 1740 cm^{-1}, and

the methylene symmetric stretching mode ($\upsilon_s CH_2$) of the hydrocarbon chains, associated to the band at ~ 2850 cm^{-1}.

Figure 4 shows the $\upsilon C{=}O$ band of nisin/DPPG system within the temperature range 20 - 60 °C. The infrared band of the carbonyl stretching in the pure DPPG lipid system at 20 and 60 °C were also included in Figure 4 for comparison. Two band components were distinguished for this vibration, located at 1739 and 1723 cm^{-1} in the spectrum of pure DPPG at 20°C and at 1741 and 1727 cm^{-1} in the nisin/DPPG system at the same temperature. Previous studies have demonstrated that the relationship between the absorbance of these individual components is a measure of the hydration of the interfacial region is sensitive to the phase-state of the lipid [32,45]. For a better appreciation of the spectral changes in the $\upsilon C{=}O$ band, Figure 4 also shows the variation of the absorbance ratio between both carbonyl components in the spectra of pure DPPG and nisin/DPPG systems as a function of temperature. In agreement with the incorporation of water molecules into the interfacial region in the L_α lipid phase, the absorbance ratio in the pure DPPG system became significantly higher at temperatures above the main transition of the lipid phase (Figure 4 B). In presence of nisin, the absorbance ratio was lower as compared to the pure lipid, both in the gel and in the liquid-crystalline phase, indicating that nisin induced a high restriction to water incorporation to the interfacial region. It was also observed that the increase in the absorbance ratio was more progressive, corresponding to a less cooperative lipid phase transition.

The $\upsilon_s CH_2$ band is a known marker of the lipid phase-state, since its position and shape are sensitive to changes in the chain order [46,47]. We compared the behavior of this band in pure DPPG vesicles and in nisin/DPPG complex in order to evaluate the effect of the peptide on the main transition temperature of the lipid membrane. Figure 5 shows the position of the $\upsilon_s CH_2$ band as a function of temperature for both DPPG systems; the derivations of the curves are included for

a better appreciation of the corresponding inflection points. A small increase in the temperature of the lipid phase transition was observed as a consequence of nisin incorporation into the bilayer. This was explained by taking into account a hydrogen-bond rearrangement, probably caused by nisin-induced dehydration in the polar region. A similar effect on the T_m was reported by a study of PG membranes upon incubation at low temperatures, which was explained in terms of the strong hydrogen-bond interactions between the headgroup hydroxyls and the phosphate moiety of adjacent PG molecules [48].

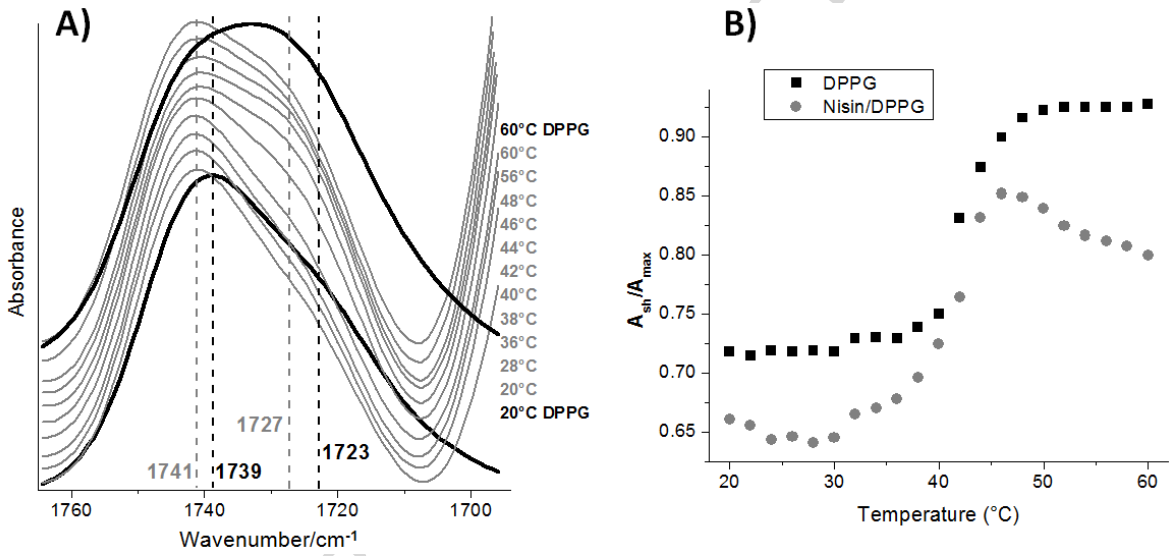

Figure 4: A) υC=O band in nisin/DPPG spectra at different temperatures between 20 and 60°C. For clarity, only selected spectra are shown. For comparison purposes, only the υC=O band of pure DPPG at 20 and at 60°C (grey traces) are included. B) Absorbance ratio between both components (A_{sh} / A_{max}) of the C=O stretching band as a function of temperature. A_{1727}/A_{1741} for nisin/DPPG and $A_{1723}/_{1739}$ for pure DPPG.

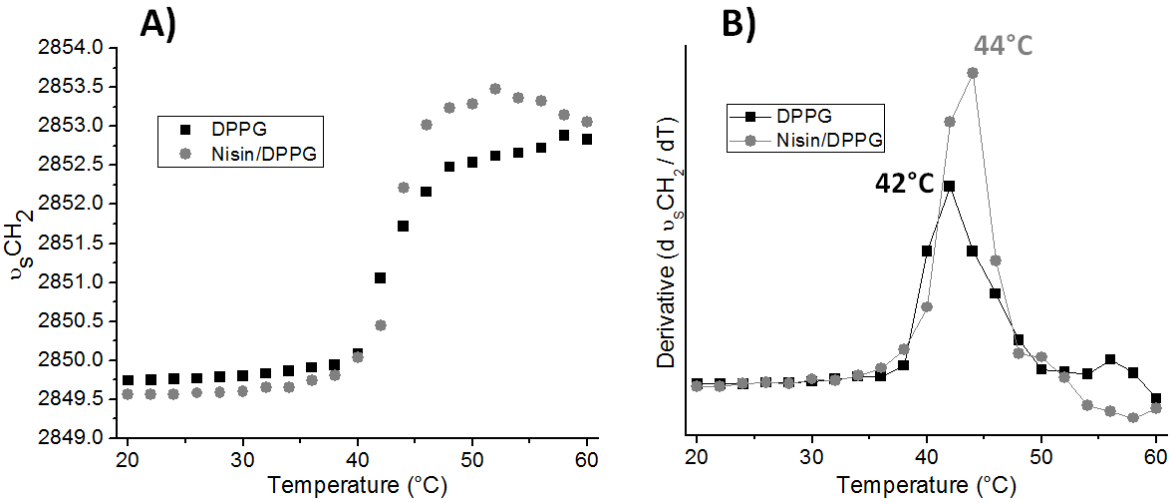

Figure 5: A) Position of the lipid $\upsilon_s CH_2$ band in pure DPPG and nisin/DPPG systems as temperature (T) increases. B) Derivative of the $\upsilon_s CH_2$ (d $\upsilon_s CH_2$ / dT) VS T for pure DPPG and nisin/DPPG systems.

3.2. Raman spectra of lipid systems. We have previously studied the structural perturbations of model membranes by the incorporation of different biomolecules using Raman spectroscopy [34, 35,49-52]. Identification, decomposition, and monitoring of specific Raman bands belonging to vibrations of PG lipid molecules allowed us to derive valuable information regarding the degree of the alterations in bilayers, both in gel and liquid-crystalline lipid phases, by the interaction with biomolecules, among them, the LII molecule [34].

Nisin effects on lipid membranes were also evaluated by Raman spectroscopy. The Raman spectra of the nisin/DPPG and nisin/DLPG systems at 20°C were compared with those of pure DPPG (L_{β}' phase) and DLPG (L_{α} phase) systems at the same temperature. In Figure 6, the spectral region comprised between 1150 and 1020 cm^{-1} of each system is shown together with the corresponding spectral decompositions. It is well known that this spectral range provides reliable information about both the interface and the hydrophobic regions of the bilayer [29, 31]. On one

hand, the Raman bands due to the C-C bond-stretching give information regarding the population of *trans*/*gauche* conformations in the acyl chains of the lipids. Typically, the two strong and well defined bands at about 1128 and 1062 cm^{-1} in the spectra of lipid membranes are associated to the in-phase and out-of-phase stretching modes of *trans* conformers, $\upsilon_{i.ph}(C\text{-}C)_T$ and $\upsilon_{o.o.ph}(C\text{-}C)_T$, respectively, while the broad and complex band of variable intensity, appearing between 1090 and 1070 cm^{-1}, is associated with the C-C stretching originated from *gauche* conformers. This last feature is normally well-resolved by band decomposition into two main components, attributed to *gauche*-*trans*-*gauche* ($\upsilon(C\text{-}C)_{G\text{-}T\text{-}G}$) and end-*gauche* ($\upsilon(C\text{-}C)_{e\text{-}G}$) conformations in the chains [34,35,51,52]. The intensity ratio between the *gauche* and *trans* bands allows to characterize the lipid phase and detect perturbations in the hydrophobic region of the bilayer. On the other hand, the symmetric stretching of the phosphate group of lipid molecules ($\upsilon_s PO_2^-$) appears between 1090 and 1105 cm^{-1} as a feature of irregular contour that can be solved into two components by band deconvolution as well. The phosphate band is of significant intensity in spectra of lipids in the gel state and almost completely overlapped by the *gauche* C-C stretching band in spectra of lipids in the liquid-crystalline state [34,35,51,52]. Based on computational predictions for the negatively charged model system $[CH_3\text{-}O\text{-}P(O)_2\text{-}O\text{-}CH_2\text{-}CHOH\text{-}CH_2OH]^-$ / xH_2O (with x=1,2) that mimics the polar region of PG membranes, the component at higher wavenumber (~1000 cm^{-1}) is associated with hydrogen-bonded phosphate groups to superficial water molecules, while the other (at ~1090 cm^{-1}) is assigned to phosphates forming a hydrogen bond with the glycerol moiety of the same lipid or of adjacent PG molecules (intra and intermolecular associations, respectively) [34]. The C-O stretching of glycerol group ($\upsilon C\text{-}O$), expected between 1105 and 1125 cm^{-1}, is normally overlapped by the $\upsilon_{i.ph}(C\text{-}C)_T$ band.

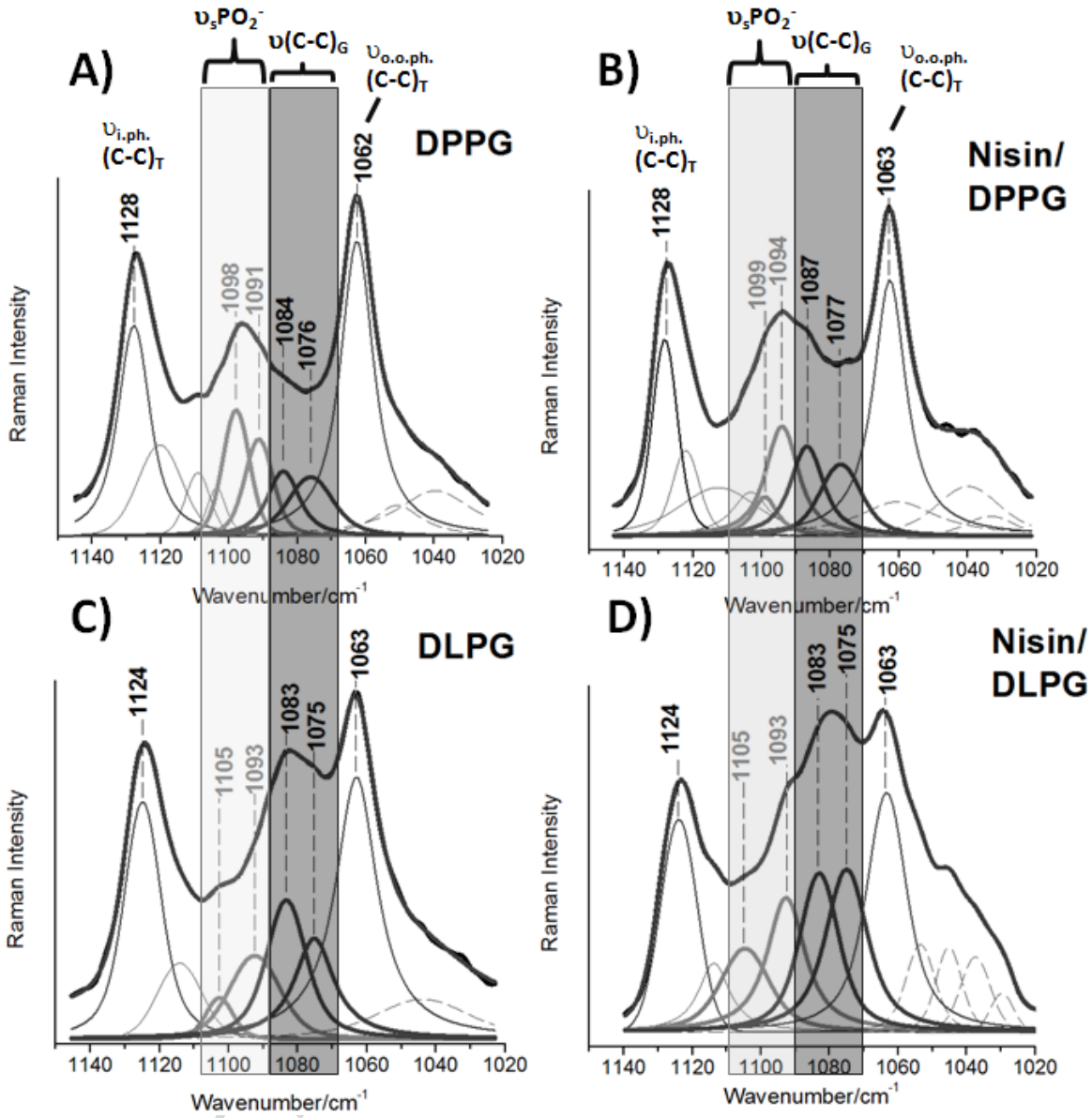

Figure 6: Band decomposition of the Raman spectral region corresponding to the C-C stretching modes of the acyl chains and to P=O stretching modes belonging to the phosphate of the lipid polar headgroups. Individual components were predicted by using a curve-fitting procedure; the reconstructed band is superimposed on the original spectrum in order to show the level of fit attained. A) pure DPPG ($L_{\beta'}$ phase); B) nisin/DPPG ($L_{\beta'}$ phase); C) pure DLPG (L_{α} phase); D) nisin/DLPG (L_{α} phase).

All the mentioned Raman features have already been identified and properly characterized in the spectra of pure DPPG and DLPG systems [34,35]. The assignment of most of the component bands predicted for the pure lipid vesicles is here presented (Figure 6, A and C) for comparative purposes with those obtained from the spectra of the nisin/DPPG and nisin/DLPG systems (Figure 6, B and D).

Spectral differences observed upon nisin incorporation to DPPG vesicles (Figure 6, A and B) were mainly evidenced by changes in relative intensities of the components associated to the phosphates and the *gauche* conformers. In the nisin/DPPG system (Figure 6 B), the dramatic intensity decrease of the $\upsilon_s PO_2^-$ component at 1099 cm^{-1} (1098 cm^{-1} in pure DPPG) confirmed the ability of the peptide to dehydrate the phospholipid headgroups of the anionic membrane, as had been demonstrated by an early 31P NMR study of nisin bound to PG bilayers [19]. In addition, the population of *gauche* conformers was increased in our nisin/DPPG system: the components at 1087 ($\upsilon(C\text{-}C)_{e\text{-}G}$) and 1077 cm^{-1} ($\upsilon(C\text{-}C)_{G\text{-}T\text{-}G}$) resulted approximately 59% and 40%, respectively, more intense than in the corresponding spectrum of pure DPPG, as was estimated from the intensity ratios between each *gauche* components and the $\upsilon_{o.o.ph}(C\text{-}C)_T$ [34,35]. This last effect may indicate that nisin was able to alter the hydrophobic region of the bilayer by introducing, at least partially, the N-terminus while the C-terminus remained anchored to the surface by electrostatic interactions, in concordance with previous studies [21-23]. However, taking into account that not significant changes in the secondary structure of nisin bound to a membrane in the gel phase (see Figure 3A), and that the peptide adopts a parallel orientation on the membrane surface [22], the structural change in the acyl lipid chains can be also interpreted as a response of the membrane to maximize the hydrophobic interactions. It has been proposed that voids in the hydrophobic core of the membrane can be created below the peptides, if they lie parallel to the bilayer plane; this

energetically unfavorable situation can be balanced by increasing the *trans*/*gauche* isomerization in those localized areas [53].

Conversely, nisin-induced structural modifications on the membrane in the liquid-crystalline phase were more drastic in the hydrophobic region of the bilayer than in the polar region (Figure 6, C and D). The apparent enhancement of both $\upsilon_s PO^{2-}$ components (1105 and 1093 cm^{-1}) in nisin/DLPG spectrum, upon comparison with that of the pure DLPG, indicated that the peptide did not induce any restriction to hydrogen bond interactions involving the phosphate groups. Instead, intensification of *gauche* conformers in the acyl chains, mainly of that assigned to $\upsilon(C\text{-}C)_{G\text{-}T\text{-}G}$, and the consequent intensity decrease of the $\upsilon_{i.ph}(C\text{-}C)_T$ and $\upsilon_{o.o.ph}(C\text{-}C)_T$ components, were remarkable. These spectral differences can be interpreted in two different ways: i) the increase of *gauche* conformations obeys to the need of the lipid system to fill the voids generated by the peptide when binding in parallel orientation to the surface; the high flexibility of hydrocarbon chains in the liquid-crystalline phase would facilitate the *trans*/*gauche* isomerization [53]. ii) The peptide molecule reached the lipid core of the bilayer, promoting a significant increase in the chain disorder. The last statement would more likely since it is in line with the structural changes observed by FTIR for nisin structure in its bound state to the membrane in the liquid-crystalline phase (see Figure 3 D), that indicated a truly hydrophobic environment surrounded most of the peptide molecule.

Results here presented show that, although the initial nisin binding to anionic membranes is not dependent on the lipid phase, its ability to penetrate the hydrophobic core, and thus form a pore, would be more limited when the lipid system is in the gel phase than when it is in the liquid - crystalline phase. As was already stated, the assays were performed at the constant 1:5 (nisin:PG) molar ratio that ensures that most of the nisin is bound to the multilamellar lipid membranes [20]. Based on previous results accounting about the ability of nisin to translocate across the bilayer by

forming transient pores [23], and/or to form pores composed by multiple nisin molecules after aggregation on the membrane surface [7,21,22], it is likely that a significant part of the peptide used in the mixtures was also bound to the inner bilayers of the liposomes. Then, in order to reach a greater understanding of the molecular bases involved in the pore formation by nisin in model anionic membranes, it is essential to determine the critical concentration of the peptide to form the pore. In this context, recent experiments by Raman spectroscopy on unilamellar vesicles of nisin/DPPG and nisin/DLPG mixtures with variable concentration of nisin, complemented with Dispersion Light Scattering (DLS) essays for the nisin/DLPG mixtures, have been performed. Those preliminary results (data not shown) showed that the most relevant spectral differences were observed for the nisin/DLPG and nisin/DPPG complexes at 300:1 and 10:1 molar ratio (lipid:peptide), respectively. Such spectral changes indicated that, at the mentioned critical concentrations of nisin, the acyl chains of the lipids underwent a decrease in the number of *gauche* conformations, with the consequent increase in the lipid packing [29]. On the contrary, peptide concentrations lower and higher than the critical concentration induced a fluidification of the bilayers. This membrane behavior would confirm the wedge model as the mechanism by which nisin is able to form pores in model membranes, as well as would demonstrate the transient nature of the pore. In addition, the preliminary results by DLS of nisin/DLPG complexes, at the same molar ratios evaluated by Raman spectroscopy, suggested that a low proportion of nisin could cause the aggregation of vesicles [19,54,55] whereas at the critical concentration, a remarkable polydispersion of the sizes of the vesicles was observed as a consequence of the formation of transient pores in these membranes [54-56]. Several new questions raised from these preliminary results that require further investigations regarding, for example, the existence of a cooperative effect of nisin on the membrane surface according to the state of the lipid phase,

the curvature and/or size variations of the unilamellar vesicles upon pore formation, among many others.

4. Conclusions

This work provides evidence of a differential effect of nisin on anionic lipid membranes, depending on the lipid phase-state. In concordance with previous reports indicating that a micromolar concentration of the peptide is necessary to penetrate model membranes [18], our nisin/DLPG system (1:5) showed strong perturbation of the hydrophobic lipid core of the membrane in the L_{α} phase, allowing us to assume that at such elevated concentrations the disruption of the bilayer may occur. However, the interactions with a lipid membrane in the $L_{\beta'}$ phase (nisin/DPPG system) indicated that the peptide interacts mainly at the superficial level by causing dehydration and/or alteration at the polar headgroups. On the other hand, structural changes were observed in nisin upon membrane penetration. A significant increase of ordered structures, especially β-turns, was observed in nisin bound to a membrane in the L_{α} phase, which is in agreement with reported structural changes of the polypeptide chain in the lipid medium [9,20].

The results here presented allow us to reach a deeper understanding, at a structural level, of the interactions that are carried out between nisin and anionic model membranes. However, further studies should be perform in model membranes containing lipids with different headgroups and/or saturation degrees in the alkyl chains that mimicking bacterial membranes, to gain insight about the peptide-lipid interactions involved in the mechanism of pore formation by this antimicrobial peptide. Moreover, the structural characterization of the PG/LII/nisin complexes

will be relevant in order to understanding the action mechanism of nisin in bacterial membranes. We concluded that the vibrational spectroscopy is a powerful tool to continue investigating the molecular bases that determine the nisin activity, especially when combining experiments by FTIR and Raman microscopy.

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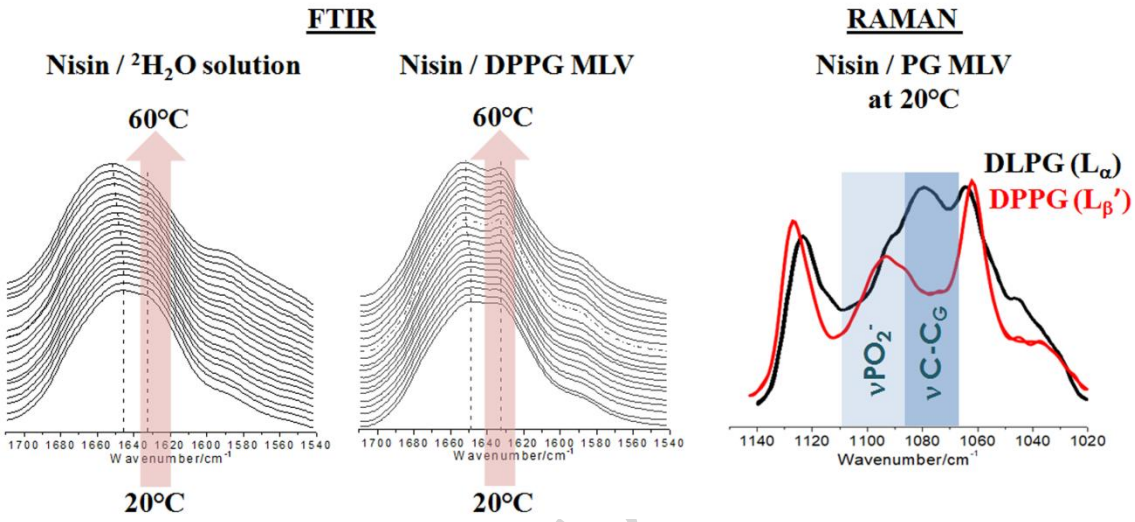
Graphical abstract



Highlights

1) The interaction of nisin with PG model membranes depends on the lipid phase-state.

2) β-turns increase in nisin bound to membranes in L_α phase.

3) Nisin dehydrates the polar headgroups in $L_{\beta'}$ phase.

4) Nisin penetrates into the hydrophobic core of the bilayers in L_α phase.

5) Vibrational spectroscopy is a powerful tool for studying peptide-lipid interactions.