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Effect of Cholesterol on the Phase Change of Lipid Membranes by Antimicrobial Peptides

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Membrane disruption by an antimicrobial peptide (AMP) was investigated by measuring the ^{2}H solid-state nuclear magnetic resonance spectra of 1-palmitoyl-d_{31}-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC_d_{31}) in mixtures of POPC_d_{31}/cholesterol and either magainin 2 or aurein 3.3 deposited on thin cover-glass plates. The line shapes of the experimental ^{2}H solid-state nuclear magnetic resonance (SSNMR) spectra were best simulated by assuming the coexistence of a mosaic spread of bilayers containing pore structures and a fast-tumbling isotropic phase or a hexagonal phase. Within a few days of incubation in a hydration chamber, an isotropic phase and a pore structure were induced by magainin 2, while in case of aurein 3.3 only an isotopic phase was induced in the presence of a bilayer phase. After an incubation period of over 100 days, alignment of the bilayers increased and the amount of the pore structure decreased in case of magainin 2. In contrast with magainin 2, aurein 3.3 induced a hexagonal phase at the peptide-to-lipid ratio of 1/20 and, interestingly, cholesterol was not found in the hexagonal phase induced by aurein 3.3. The experimental results indicate that magainin 2 is more effective in disrupting lipid bilayers containing cholesterol than aurein 3.3.

Key Words : Magainin 2, Aurein 3.3, Pore, Hexagonal, ^{2}H Solid-state NMR

Introduction

Antimicrobial peptides are an immunological biomolecule found in various organs of living species.[1-6] The antimicrobial peptides lyse the cell membrane by binding on the membrane surface.[7,8] Many factors such as the geometrical structure of the peptide,[9] the surface concentration and charge density of the peptide,[10] and the surface charge density and hydration level of the lipid membranes[11-13] are associated with the various membrane-distuption mechanisms, namely the micellization model, the carpet model, the membrane thinning model, the toroidal pore model, and the barrel-stave model.[14-16] For example, alamethicin in the fungus Trichoderma viride causes thinning of the lipid membrane at a specific concentration without lysing the membrane.[17] TAT, a HIV-derived cell-penetrating peptide that has many positively charged aminoic residues, causes a lipid membrane of zwitterionic dimyristoylphosphatidylcholine to form inverted micelles, but it does not cause a negatively-charged membrane of dimyristoylphosphatidylglycerol to form inverted micelles.[18]

Eukaryotic cells have a cholesterol-rich cell membrane whereas, in general, prokaryotic cells have a cholesterol-absent cell membrane. Also the cell membrane of eukaryotic cells has an electrically neutral surface composed of zwitterionic lipids, while the cell membrane of prokaryotic cells has an electrically negative surface composed of negatively-charged lipid molecules. These differences have a greater effect on AMP action on the cell membrane than any other factors. The presence of cholesterol attenuates membrane intercalation of an antimicrobial peptide.[19,20] The mobility of water molecules on POPC multilamellar vesicles in the presence and absence of cholesterol was investigated to study the effect of cholesterol on POPC bilayers.[21]

To investigate the effect of cholesterol on the lipid membrane phase change induced by an AMP we used two antimicrobial peptides, magainin 2 and aurein 3.3. Magainin 2 has 23 residues (GIGKFLHSAKKFGKAFVGEIMNS) and shows a wide range of antibiosis.[22] Although magainin 2 at low concentrations causes a thinning of a lipid bilayer by being bound on the membrane surface with the helical axis of the peptide parallel to the membrane surface,[15,23,24] above a critical concentration it is known to cause a pore on the lipid bilayer by being bound in the membrane with the helical axis of the peptide perpendicular to the membrane surface.[13,25-27]

Aurein is an antimicrobial peptide secreted from the granular gland of the Green and Golden Bell Frog, Litoria aurea, and the Southern Bell Frog, L. raniformis.[28] Aurein 3.3 consists of 17 residues (GLFDIVKKIAGHIVSSI) and is known to have an α-helical structure when it binds to the lipid membrane.[29] It has a similar structure to magainin 2 and is known to possess antibacterial and antitumor functions,[28] but its detailed and specific action mechanism is not well known.

Many methods have been used to investigate the action of antimicrobial peptides on membrane surfaces. A micelle[30] or a vesicle[31] has been used to simulate a biomembrane and, recently, a bicelle[32] with a flat surface is widely used. Spectroscopic methods such as circular dichroism,[33] neutron diffraction,[25] X-ray diffraction,[34] and nuclear magnetic resonance spectroscopy[35,36] have been used to analyze the action modes and the binding structure of AMPs. For example, the binding pattern of Psd1 and partition to lipid membranes containing ergosterol or cholesterol were studied using fluorescence

spectroscopy.[37] Nuclear magnetic resonance spectroscopy has been widely used to investigate the structures of insoluble peptides because it has the advantage that the spectra of a variety of nuclei can be measured to obtain much information.[38,39]

In the present paper we studied the phase change of POPC_d_{31} lipid bilayers after incubation periods in the presence of α-helical structured antimicrobial peptides by using the SSNMR spectra. In the previous papers, we had investigated the phase change of POPC lipid or POPC/POPG lipid mixtures induced by antimicrobial peptides such as protegrin-1, magainin 2, and aurein 3.3. Here, we investigated the cholesterol effect on the phase changes of lipid-peptide mixtures with incubation times over 100 days. At initial time two peptides showed a similar effect on the lipid phase but after 100 days of incubation the peptide effects were well distinguished.

Experimental

Materials. 1-Palmitoyl-d_{31}-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC_d_{31}) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Magainin 2 and aurein 3.3 were purchased from GL Biochem (Shanghi, China) and used without any further purification. Trifluoroethanol (TFE), chloroform, cholesterol, and sodium phosphate dibasic were purchased from Aldrich Chemicals (Milwaukee, WI, USA). Thin cover-glass plates with a size of 80 μm × 10 mm × 10 mm were purchased from Marienfeld Laboratory Glassware (Bad Mergentheim, Germany).

NMR Sample Preparation. Mechanically aligned lipid bilayers were prepared on thin cover-glass plates to produce a biomembrane mimic system by following the standard procedure developed by Hallock *et al*.[13] The peptides were dissolved in TFE and mixed with chloroform solution containing POPC_d_{31} and cholesterol (POPC_d_{31} : cholesterol = 1 : 1). The peptide-to-lipid (P/L) molar ratios were adjusted to 1/80, 1/50, and 1/20.

The homogeneously mixed solutions were deposited onto each cover-glass plate at a surface concentration of 0.01–0.04 mg/mm^2, dried for 4 hours in air, and then vacuum-dried overnight to remove thoroughly any residual organic solvents. The samples were hydrated directly with water drops of about 2 μl[29,39] using a micropipette and placed for 2 days in a chamber with 95% relative humidity maintained by using a saturated sodium phosphate dibasic solution. 10 thin cover-glass plates containing the hydrated peptides and lipids were stacked, wrapped in Parafilm, and sealed in a polyethylene bag to prevent them from being dried during the NMR measurements. After a few days of incubation in a refrigerator at 5 °C, the samples were inserted into a rectangular NMR probe coil and their static ^{2}H SSNMR spectra were measured. After the first NMR spectra measurements had been taken, the samples were stored in a refrigerator at 5 °C for about 100 days and the second NMR spectra measurements were taken.

Solid-state ^{2}H Nuclear Magnetic Resonance Spectroscopy. The SSNMR experiments were performed with a Bruker Avance II 300 MHz spectrometer operating at the resonance frequencies of 46.07 MHz for ^{2}H installed at the Virginia Tech Department of Chemistry. A static double-resonance rectangular coil with the dimensions of 18 × 10 × 5 mm^3 was used for measuring the static ^{2}H NMR spectrum. The spectra were acquired using a quadrupolar echo sequence with an echo delay time of 30 μs and a recycle delay of 0.5 s. The 90° pulse length of 5.0 μs was used. The spectral width was 100 kHz. The spectra were averaged over 12,000 scans. All experiments were carried out at 20 °C.

Theoretical Considerations. The ^{2}H SSNMR spectrum has been an ideal tool for investigating the geometrical distribution of lipids forming biomembranes.[28,40] In order to obtain a credible structure and dynamical parameters, simulation of the experimental spectra should be carried out properly and precisely. Here, we omit the detailed procedure because the proper treatment was presented in previous papers.[41] While many different types of structural phases can coexist in lipid-peptide mixtures, in order to simplify the situation we assumed the pore formation by magainin 2 as this has been well proven in many other papers.[13,25,27,28] Also it was assumed that the pore structure had a circular toroidal geometry to increase the fidelity of the parameters determined through the computer simulation. In case of aurein 3.3, we did not include the pore structure because a good simulation of the ^{2}H SSNMR spectra could be provided by the mixture of an isotropic phase and a well-aligned bilayer phase, except for the case with the P/L ratio of 1/20. In case of the P/L ratio of 1/20, it was necessary to apply the hexagonal phase to the geometry simulation because no other structure could offer an appropriate simulation of the ^{2}H SSNMR spectrum.

Results and Discussion

Phase Changes of POPC_d_{31}/Cholesterol Bilayers Induced by Magainin 2. It is well known that magainin 2 induces a pore structure in the lipid bilayer[13] even in the presence of cholesterol. The ^{2}H SSNMR spectra of the mixture of magainin 2, POPC_d_{31}, and cholesterol were simulated using a model composed of both isotropic vesicles and well-aligned bilayers containing pore structures. It is very obvious that the sharp peaks at 0 ppm in Figure 1(i) and 1(k) were originated from the isotropic phase such as micelles or vesicles. The pore formation in the well-aligned multi-bilayers was assumed because many other papers proposed that formation. The other phases were not considered because the experimental spectra were well fitted to the simulation results assuming three phases.

The results are shown in Figure 1 and in Table 1 The phases of the mixture of POPC_d_{31} and magainin 2 in the presence of cholesterol changed as the incubation time increased from within 10 days to more than 100 days, which is very similar to the results obtained in the previous paper using POPC_d_{31} or POPC_d_{31}/POPG membranes without cholesterol.[40]

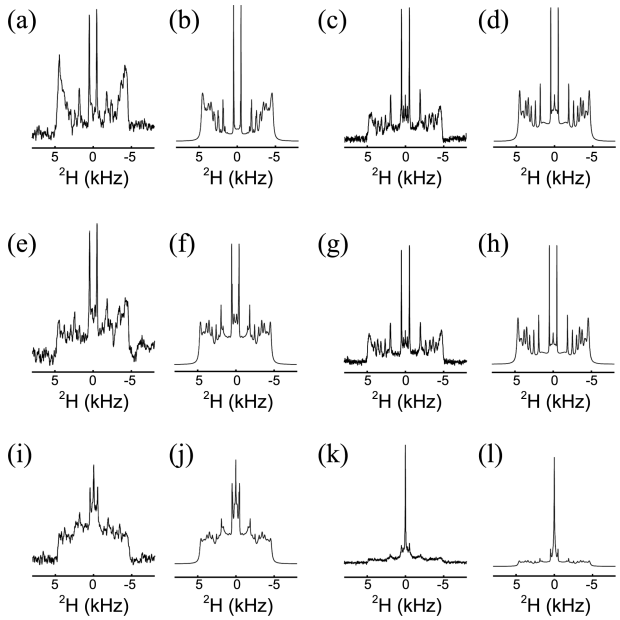

Figure 1. Experimental (a, c, e, g, i, and k) and simulated (b, d, f, h, j, and l) ^{2}H SSNMR spectra of POPC_d_{31} in POPC/cholesterol membranes disturbed by magainin 2. P/L ratios are 1/80 (a-d), 1/50 (e-h), and 1/20 (i-l). Spectra in (a), (e), (i) were measured within a few days of incubation after hydration and those in (c), (g), (k) were measured after an incubation period of over 100 days. Simulated spectra were obtained by assuming 3 phases of an aligned bilayer, an isotropic phase, and a pore structure. The percentages of lipid involved in 3 phases are shown in Table 1.

In the initial state, the portion of the well-aligned bilayers was small, but the portion increased after a period of over 100 days. On the contrary, the portion of pore structures decreased as the incubation time increased except in case of the 1/80 P/L ratio. The reason for this is believed to be as follows: at the initial time, the bilayers were not aligned on the thin glass plate surface and the surface normal axis of the small-sized bilayers was randomly distributed.

The random distribution of the small-sized bilayers could be simulated as a pore structure,[41] but after a period over 100 days the bilayers were well aligned on the thin-glass plate surface and the pore structure was also well-aligned. This phenomenon can be explained as follows: much time is needed for the peptides, which are evenly distributed at the initial time, to travel in the lipid-peptide mixture and then to self-aggregate in order to induce a well-defined pore structure or an isotropic phase such as a micelle or vesicle. This is supported by the appearance of a large amount of the isotropic phase after a period of over 100 days. The slow phase change is attributed to the physical characteristic of our sample prepared on the glass plates. In our sample, the bilayers are multilayered with a small distance of the water interface so that because of a strong interaction between the layers the lipid bilayers are more rigid than the unilamellar layer in aqueous vesicles. In this situation the peptides cannot move as fast as in the unilamellar bilayer in aqueous solution.

Table 1. Percentages of 3 phases of POPC_d_{31}/cholesterol induced by magainin 2

P/L ratio	1/80		1/50		1/20	
Phases	Storage time (days)					
	< 10	> 100	< 10	> 100	< 10	> 100
Isotropic	0%	1%	0%	1%	3%	26%
Bilayer	75%	45%	30%	55%	15%	20%
Pore	25%	54%	70%	44%	82%	54%

A dramatic change was found in the sample with a P/L ratio of 1/20. After a period of over 100 days, the isotropic phase increased to 26% and well-aligned bilayers decreased to 20%. This indicates that a low concentration of magainin 2 induced a pore structure through peptide aggregation in the bilayer, but at a higher peptide concentration the larger number of peptide aggregations resulted in the complete destruction of the bilayer.

As expected, the relative amount of pore structures in the POPC_d_{31}/cholesterol bilayer was smaller than that in POPC_d_{31} or POPC_d_{31}/POPG bilayers. The pore percentage was 60% in the POPC_d_{31} bilayers, 80% in the POPC_d_{31}/POPG bilayers, and 54% in the POPC_d_{31}/cholesterol bilayers, but the effect of cholesterol on the formation of pore structures was less than expected.

By simulating the ^{2}H spectra, quadrupolar coupling constants (QCC) of ^{2}H nuclei were obtained and then the order parameters were calculated with the reference value of 168 kHz for the static ethylene ^{2}H nucleus.[42] The order parameters were a little smaller than those for the pure POPC_d_{31}/cholesterol bilayers, which means that the insertion of the peptides into the bilayers enables the acyl chains to move more freely than in the POPC_d_{31} bilayer/cholesterol bilayers.

Phase Changes of POPC_d_{31}/Cholesterol Bilayers Induced by Aurein 3.3. The phase change of the aurein 3.3-lipid mixture was quite different to the case of magainin 2. The pore structure was not assumed to be formed by aurein 3.3, which is consistent with the facts that the antimicrobial peptide aurein 1.2 disrupts model membranes via the carpet mechanism[43] and that the binding angle of aurein 2.3 is oblique to the membrane surface.[44]

On the contrary, an isotropic phase was formed at the high P/L ratio of 1/20 after over 100 days incubation. The structure of the isotropic phase had a much smaller rotational diffusion coefficient than that in the case of magainin 2, which means that the lateral diffusion of the lipid molecules in the isotropic phase was much inhibited by aurein 3.3 or the overall rotational motion of the structure was very slow because of the larger size of the isotropic phase; however, as the incubation time increased the isotropic phase decreased and the bilayer phase increased.

An interpretation for this is that the lipid phase was randomly distributed at the initial time because of evenly distributed peptide molecules among the lipid molecules. This random distribution could be simulated as an isotropic phase; but, at some time later the randomly distributed peptides aggregated and produced a small portion of a true isotropic

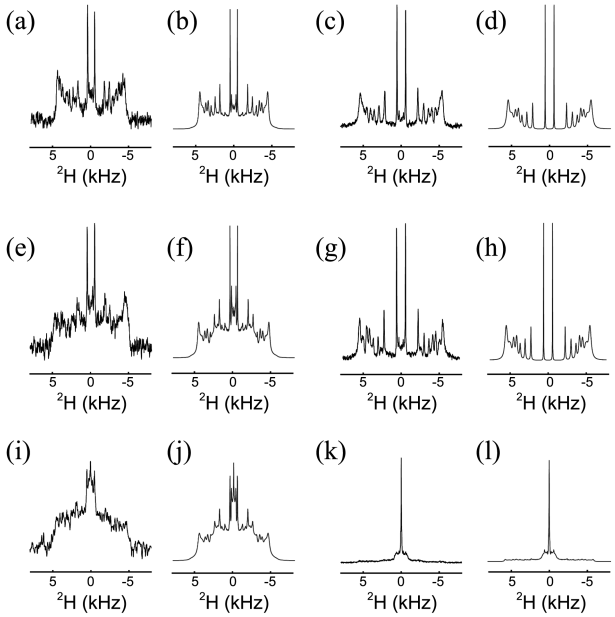

Figure 2. Experimental (a, c, e, g, i, and k) and simulated (b, d, f, h, j, and l) ^{2}H SSNMR spectra of POPC_d_{31} in POPC/cholesterol membranes disturbed by aurein 3.3. P/L ratios are 1/80 (a-d), 1/50 (e-h), and 1/20 (i-l). Spectra in (a), (e), (i) were measured within a few days of incubation after hydration and those in (c), (g), (k) were measured after an incubation period of over 100 days. Simulated spectra were obtained by assuming 3 phases of an aligned bilayer, an isotropic phase, and a hexagonal phase. The percentages of lipid involved in 3 phases are shown in Table 2.

Table 2. Percentages of 3 phases of POPC_d_{31}/cholesterol induced by aurein 3.3

P/L ratio	1/80		1/50		1/20	
Phases	Storage time (days)					
	< 10	> 100	< 10	> 100	< 10	> 100
Isotropic	50%	0%	70%	0%	74%	20%
Bilayer	50%	100%	30%	100%	26%	50%
Hexagonal	0%	0%	0%	0%	0%	30%

phase while, in the absence of aurein 3.3, a large portion of the lipid molecules produced well aligned bilayers. In contrast with the low P/L ratios, at the P/L ratio of 1/20 30% of the hexagonal phase was formed after a period over 100 days. This hexagonal phase was also found in the POPC_d_{31}/POPG lipids.[40] As shown in Figure 3, the line shape of the ^{2}H SSNMR spectrum was best fitted with 20% of the isotropic phase, 50% of the bilayer phase, and 30% of the hexagonal phase. The existence of the hexagonal phase was confirmed by the fact that the broad peak at the center of the spectrum could not be fitted with any other geometrical structure such as isotropic vesicle, well-aligned bilayer, toroidal pore, or membrane thinning. At a sufficient concentration of aurein 3.3 on the lipid membrane surface the hexagonal phase was formed in the POPC_d_{31}/cholesterol, but the portion of the phase was less than the case of POPC_d_{31}/POPG, which had 70% of the hexagonal phase.

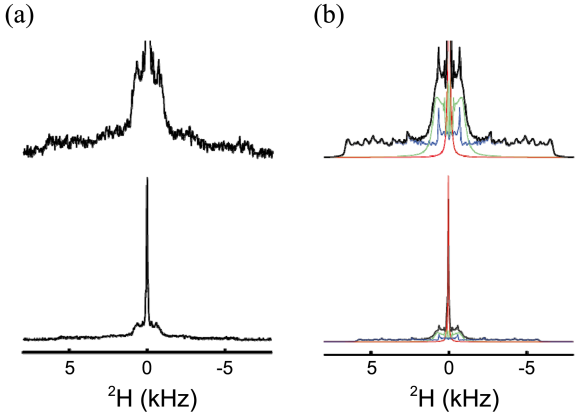

Figure 3. Experimental (a) and simulated (b) ^{2}H SSNMR spectra of POPC_d_{31} in POPC/cholesterol membranes disturbed by aurein 3.3 at the P/L ratio of 1/20. The top spectrum shows a good simulation of the broad part of the full spectra shown in the bottom. Simulated spectrum was obtained by assuming 3 phases of an aligned bilayer (blue), an isotropic phase (red), and a hexagonal phase (green). The percentages of lipid involved in 3 phases are shown in Table 2.

It is interesting to note that the QCC values of ethylene ^{2}H of POPC_d_{31} in the hexagonal phase are a little larger than those of the pure POPC_d_{31} bilayers without cholesterol. Figure 4 shows the order parameters of ethylene units of the POPC_d_{31} molecule in the POPC_d_{31} bilayer, POPC_d_{31}/cholesterol bilayer, and POPC_d_{31} hexagonal phase in the presence of aurein 3.3. The order parameters calculated from the QCC values indicate the reorientational mobility of the ethylene units. In general, the inclusion of cholesterol molecules reduces the mobility of ethylene units of POPC_d_{31} and results in an increment of about two times of the order parameters. The order parameters of the ethylene units in the hexagonal phase show that the hexagonal phase of POPC_d31 formed by aurein 3.3 does not have cholesterol. In case the amount of aurein 3.3 molecules deposited into the cholesterol-POPC_d_{31} bilayers exceeds a threshold, the lipid-bound aurein 3.3 excludes cholesterol from the POPC_d_{31} bilayer and then produces a hexagonal phase composed of POPC_d_{31} and aurein 3.3. The small increment of the order parameters in the [aurein 3.3 + POPC_d_{31}] hexagonal phase compared with those in the pure POPC_d_{31} bilayer phase indicates that the inserted aurein 3.3 molecules decreased the reorientational mobility of the acyl chain.

Comparison Between Two Peptides. Two peptides showed a similar tendency, but there was a big difference between them at the P/L ratio of 1/20. As the incubation time increased, the bilayer aligned very well and showed characteristic structures. A pore structure was well defined with magainin 2, while a hexagonal phase was formed with aurein 3.3. This main difference between the lipid structures formed by magainin 2 and aurein 3.3 may be because the two peptides have alpha helical structures of different lengths. Magainin 2 consists of 23 residues with a helical length of 34.5 Å.

The distance between the phosphate groups in the POPC_d_{31} bilayer is 35 Å, and the distance between the choline groups

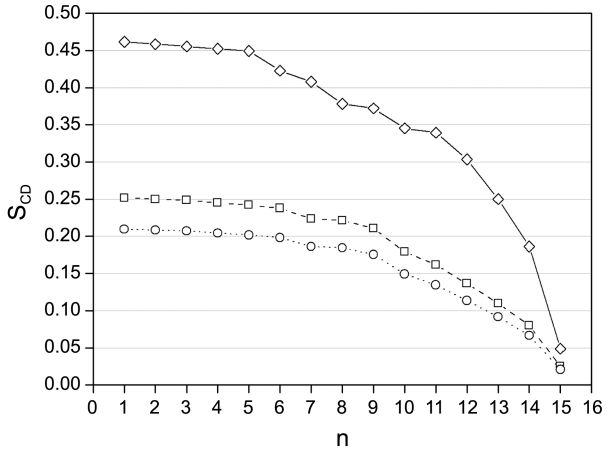

Figure 4. The orientational order parameter (S_{CD}) profiles of acyl chain in POPC_d_{31}. A pure POPC_d_{31} bilayer (○), a POPC_d_{31}/cholesterol bilayer (◇), and the hexagonal phase induced by aurein 3.3 in the presence of cholesterol (□).

is 38 Å.[45] This match between the length of the magainin 2 helix and the distance between the phosphate groups in the POPC_d_{31} bilayer enables effective hydrophobic and hydrophilic interactions between the peptide and lipid molecules in case the peptides insert into the bilayer and reside at a perpendicular orientation to the bilayer. In contrast, aurein 3.3 consists of 17 residues and has a helical length of 25.5 Å.

This mismatch between the length of the aurein 3.3 helix and the distance between the phosphate groups in the POPC_d_{31} bilayer causes aurein 3.3 to bind at an oblique angle to the bilayer and have difficulty in producing the pore structure.

Conclusion

Structural change of a POPC_d_{31}/cholesterol bilayer was measured in the presence of an antimicrobial peptide, either magainin 2 or aurein 3.3, by using the ^{2}H SSNMR technique. A big difference was found between the phases formed within 10 days and after over 100 days of incubation. Within 10 days of incubation, there appeared to be large portions of the isotropic phase or pore structure, but the portion of these phases decreased after over 100 days. At the time the lipid-cholesterol-peptide mixtures were deposited on the thin glass plate surface, the bilayer normal was randomly distributed, but as the incubation time increased the size of the lipid bilayer increased and was well aligned on the glass plate surface. Consequently, the large portion of isotropic phase or pore structure in the mixture within a few days of incubation appears to be the result of a misinterpretation of the randomly orientated bilayers not being aligned on the glass plate surface.

As with the cases of POPC_d_{31} or POPC_d_{31}/POPG, magainin 2 caused a pore structure to develop in the POPC_d_{31}/cholesterol bilayer at all the P/L ratios. In contrast with magainin 2, aurein 3.3 had no effect on the disruption of the POPC_d_{31}/cholesterol bilayer at low P/L ratios, but at the higher P/L ratio of 1/20 an isotropic phase and a hexagonal phase appeared after over 100 days of incubation. These results indicate that magainin 2 is more effective in disrupting lipid bilayers containing cholesterol than aurein 3.3.

The result of greatest interest is that cholesterol molecules were not found in the hexagonal lipid phase induced by aurein 3.3 at the P/L ratio of 1/20. Cholesterol was only present in the well-aligned bilayer phase that had not been disrupted by aurein 3.3. This shows that the antimicrobial peptide aurein 3.3 excludes the cholesterol molecules from the lipid bilayer in order to disrupt effectively the lipid membrane.

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