The Effects of Gramicidin on the Structure of Phospholipid Assemblies

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ABSTRACT   Gramicidin is an antibiotic peptide that can be incorporated into the monolayers of cell membranes. Dimerization through hydrogen bonding between gramicidin monomers in opposing leaflets of the membrane results in the formation of an iontophoretic channel. Surrounding phospholipids influence the gating properties of this channel. Conversely, gramicidin incorporation has been shown to affect the structure of spontaneously formed lipid assemblies. Using small-angle x-ray diffraction and model systems composed of phospholipids and gramicidin, the effects produced by gramicidin on lipid layers were measured. These measurements explore how peptides are able to modulate the spontaneous curvature properties of phospholipid assemblies. The reverse hexagonal, H_{II}, phase formed by dioleoylphosphatidylethanolamine (DOPE) monolayers decreased in lattice dimension with increasing incorporation of gramicidin. This indicated that gramicidin itself was adding negative curvature to the lipid layers. In this system, gramicidin was measured to have an apparent intrinsic radius of curvature, R_{0p}^{gram}, of -7.1 Å. The addition of up to 4 mol% gramicidin in DOPE did not result in the monolayers becoming stiffer, as measured by the monolayer bending moduli. Dioleoylphosphatidylcholine (DOPC) alone forms the lamellar (L_α) phase when hydrated, but undergoes a transition into the reverse hexagonal (H_{II}) phase when mixed with gramicidin. The lattice dimension decreases systematically with increased gramicidin content. Again, this indicated that gramicidin was adding negative curvature to the lipid monolayers but the mixture behaved structurally much less consistently than DOPE/gramicidin. Only at 12 mol% gramicidin in dioleoylphosphatidylcholine could an apparent radius of intrinsic curvature of gramicidin (R_{0p}^{gram}) be estimated as -7.4 Å. This mixture formed monolayers that were very resistant to bending, with a measured bending modulus of 115 kT.

INTRODUCTION

The vast repertoire of membrane function results from the complex composition and assembly of proteins and lipid species. The simple bilayer architecture contains many molecular species that spontaneously assemble into curved, nonbilayer structures when isolated and hydrated (Luzzati and Husson, 1962). These assemblies can be conceptualized as resulting from packing the projected shapes of the lipid molecules, where cylindrical lipids form the flat lamellar phase (L_α) and cone-shaped lipids form hexagonal phases with highly curved monolayers, reviewed by (Cullis and de Kruijff, 1979). The reverse hexagonal phase (H_{II}) consists of indefinitely long tubes filled with water (Fig. 1).

Non-bilayer-prone lipids are thought to play a key role in reducing the energetic barriers of forming highly curved lipidic intermediates in the process of membrane fusion, reviewed by (Chernomordik et al., 1995). In addition, membrane protein conformations and activity are sensitive to the composition of their surrounding lipids, and many are affected by non-bilayer-prone lipids. Bilayers containing components that do not themselves pack into a flat surface, are likely to be stressed in a way that affect these two fundamental processes through lipid-lipid and lipid-protein interactions.

Quantifying the stresses introduced by individual lipid species can be achieved by studying purified lipids in model systems. When allowed to form unstressed assemblies, lipids form curved structures as a manifestation of their intrinsic curvature (Gruner, 1985). The intrinsic curvature and bending moduli of several lipids and mixtures of lipids have been measured (Chen and Rand, 1997; Epand et al., 1996; Leikin et al., 1996; Rand et al., 1990). Indeed lipid curvature has been shown to affect the gating properties of the peptide alamethicin (Keller et al., 1993; Bezrukov, 2000).

On the other hand the contribution of proteins to the curvature of membranes, important functionally (Bezrukov, 2000), has not yet been measured. Certainly it has been shown that peptides contribute to the phase structure of membrane lipids (Lindblom et al., 1988) and it has been shown that alamethicin induces the cubic phase (Keller et al., 1996). Here we attempt to quantify the curvature contribution of a peptide.

Gramicidin is a membrane peptide with antibiotic and channel-forming properties first isolated by Dubos (1939). We have used it in this first attempt to measure a protein's contribution to membrane curvature properties. It is small, can be easily modified, and its function is well documented. It has provided a model for i), the effects of peptide sequence on three-dimensional structure (Andersen et al., 1996), ii), the structure-function relationship of an iontophoretic channel (Koeppe and Andersen, 1996), (Busath, 1993), and iii), for lipid-protein interactions. Gramicidin has been used for studying the effects of the lipids on channel function (Andersen et al., 1998), and the effects of peptide inclusion on the packing properties of lipids (Killian, 1992). It has been shown to induce a transition from flat to nonflat lipid structures in model systems (Killian et al., 1986; Tournois et al., 1987; Van Echteld et al., 1982; Van Echteld et al.,
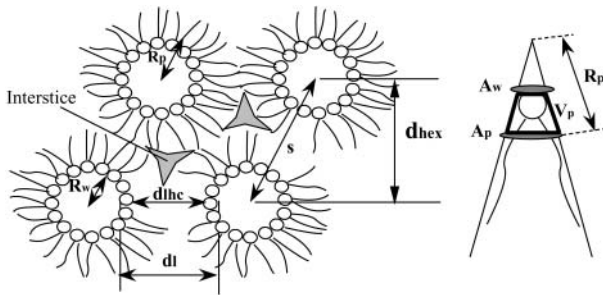
FIGURE 1 Schematic representation of the reverse hexagonal phase with the structural parameters shown. These variables are described and defined in the text.

1981). In this study we attempt to measure its intrinsic curvature and its effect on the membrane bending modulus, to aid estimates of its effect on membrane deformation energies (Helfrich and Jacobsson, 1990; Nielsen et al., 1998).

MATERIALS AND METHODS

Sample preparation

All phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs) were purchased from Avanti Polar Lipids (Birmingham, AL) and stored under nitrogen at $-18°C$. Gramicidin D was purchased from Sigma Chemical Co. (St. Louis, MO) and stored under nitrogen at $2°C$. Tetradecane (td) was purchased from Sigma Chemical Co. and stored at room temperature. Hereafter we refer to gramicidin D as gramicidin. Fluka Chemika polyethylene glycol 20,000 (PEG) crystals were purchased from Caledon Laboratories Ltd. (Georgetown, ON) and stored at room temperature. PEG solutions were prepared by mixing the desired weight of PEG crystals with double distilled water.

Appropriate dry weights of phospholipid and gramicidin D were dissolved in 3:1 chloroform:methanol and mixed together in solution. The solvent was removed by rotary evaporation followed by desiccation under vacuum. For selected samples, tetradecane was added to make up 16% of the dry weight of the total lipid mixture and allowed to equilibrate for 72 h at room temperature. Appropriate proportions of double-distilled water were added to the lipid mixtures by weight fraction and allowed to equilibrate for ~5 days. Alternatively, hydration was achieved by immersing the lipid mixtures in ~3 mL PEG solution and equilibrating for ~5 days. After equilibration, the samples were examined by x-ray diffraction. Teflon shavings, providing an x-ray calibration line at 4.87 Å, and the hydrated lipid mixtures were placed into x-ray sample holders and sealed between mica windows 1 mm apart.

X-ray diffraction

A Rigaku rotating anode was used to generate x-rays where the $CuK_{\alpha 1}$ line (wavelength of 1.540 Å) was isolated using a bent quartz crystal monochromator. The diffraction patterns were captured on film using Guinier x-ray cameras. Thermoelectric elements were used to maintain the desired temperature to $\pm 0.5°C$.

The x-ray diffraction pattern was used to characterize the phase formed by the lipid and its lattice dimension. Hexagonal phases gave spacings in the ratios of 1, $1/\sqrt{3}$, $1/\sqrt{4}$, $1/\sqrt{7}$, $1/\sqrt{9}$, $1/\sqrt{12}$, etc. which were used to measure the lattice dimension d_{hex}. The lamellar phase gave spacings in the ratios of 1, 1/2, 1/3, 1/4, etc. giving the lattice dimension d_{lam}. The coexistence of independent sets of spacings indicated the coexistence of phases within the sample.

Experimental systems

The fully hydrated phases formed by increasing concentrations of gramicidin in dioleoylphosphatidylethanolamine (DOPE) and in dioleoyl-phosphatidylcholine (DOPC) were observed. This survey was performed on DOPE systems, without td, and DOPC systems, with td, to determine the composition range that would allow further structural analysis.

Phase diagrams relating lattice dimension to water content were constructed for mole fractions of 0.00, 0.01, 0.02, 0.03, and 0.04 gramicidin in DOPE. Increasing amounts of water were added gravimetrically to samples of each composition. Phase diagrams were also constructed for mole fractions of 0.07, 0.08, 0.10, 0.12, and 0.14 gramicidin in mixtures with DOPC in the presence of td.

Sensitivity of the lattice dimensions to osmotic stress was measured for mole fractions of 0.00–0.04 gramicidin in mixtures with DOPE, and mole fractions of 0.07–0.14 gramicidin in mixtures with DOPC, in the presence of td. Various concentrations of PEG 20,000 were used to exert known osmotic stresses on the samples (Parsegian et al., 1986).

STRUCTURAL ANALYSIS

H_{II} phases are two-dimensional hexagonal lattices formed by the axes of indefinitely long and parallel regular prisms. Water cores, centered on the prism axes, are lined with the lipid polar groups, and the rest of the lattice is filled with the hydrocarbon chains.

For a hexagonal phase of known composition, the measured lattice can be divided into compartments, as shown in Fig. 1, each containing defined volume fractions of the lipid, peptide and water. This volume average division follows the method originally introduced by Luzzati (Luzzati and Husson, 1962) and depends only on the assumption of their linear addition. Specific data for the lipid components used in these systems can be found in Fuller and Rand (2001). Gramicidin's MW and density are taken as 1884 and 1.20 gm/cc.

In particular, we separate the water and nonwater compartments in the hexagonal lattice by an idealized cylindrical interface of radius R_w that encloses a volume equal to the volume of water in the H_{II} phase (Fig. 1). This dividing surface is referred to as the Luzzati plane.

The radius of the water cylinder, R_w, is related to the first-order Bragg spacing in the hexagonal phase, d_{hex}, and to the volume fraction of water in the sample, ϕ_w, as follows:

$$R_W = d_{hex}\sqrt{\frac{2\phi_w}{\pi\sqrt{3}}}. \tag{1}$$

The area per effective molecule at the Luzzati plane is given by

$$A_w = \frac{2\phi_w V_1}{(1 - \phi_w)R_w}, \tag{2}$$

where V_1 is the volume of an effective lipid molecule and

$$\phi_w = \frac{V_w}{V_w + V_1}, \tag{3}$$

where V_w is the volume of water added per effective lipid molecule in each sample.

For these calculations, we use the notion of an effective molecule that is one phospholipid + x gramicidin + y tetradecane molecules, where x is the molar ratio of gramicidin to phospholipid, and y is the molar ratio of tetradecane to phospholipid in the samples. The effective lipid molecular volume is

$$V_l = V_{pl} + xV_{gramicidin} + yV_{tetradecane}. \qquad (4)$$

V_{pl}, $V_{gramicidin}$, and $V_{tetradecane}$ are the molecular volumes of DOPE, gramicidin, and tetradecane.

Elastic energy of the hexagonal phase

The monolayers of the H_{II} phase can be described in terms of curvature, $1/R_p$, measured at a pivotal plane where the molecular area remains constant (Rand et al., 1990; Leikin et al., 1996). The elastic free energy, F, of the hexagonal phase (normalized per effective molecule) can be approximated by the energy of bending (Helfrich, 1973, Kirk et al., 1984)

$$F = \frac{1}{2} K_{cp} A_p \left(\frac{1}{R_p} - \frac{1}{R_{0p}} \right)^2, \qquad (5)$$

where K_{cp} is the bending modulus, and A_p and R_{0p} are the molecular area and the spontaneous radius of curvature at the pivotal plane.

The goal of this study is to find the position of any pivotal plane, and then measure the spontaneous curvature, molecular area, and bending moduli for different phospholipid/gramicidin mixtures. These structural parameters and elastic moduli (Leikin et al., 1996) are determined as follows:

1. The molecular area, A, and radius of curvature, R, at any cylindrical dividing surface, separated by a volume V per lipid molecule from the Luzzati plane (Fig. 1) are related by

$$A^2 = A_w^2 + 2V \frac{A_w}{R_w} \ldots .or \ldots A = A_w \sqrt{1 + \frac{(1 + \phi_w)}{\phi_w} \frac{V}{V_l}}, \qquad (6)$$

$$R = R_w \sqrt{1 + \frac{(1 + \phi_w)}{\phi_w} \frac{V}{V_l}}. \qquad (7)$$

2. We verify whether the system has a well-defined pivotal plane by plotting A_w^2 versus A_w/R_w, from Eq. 6, in the form

$$A_w^2 = A_p^2 - 2V_p \frac{A_w}{R_w}. \qquad (8)$$

If the plot is a straight line, the system has a dividing surface of constant area, the pivotal plane. The slope and the intercept of the plot give both the position of the

pivotal plane, V_p, which is the volume separating this plane and the Luzzati plane, and the molecular area of an effective molecule at that plane, A_p.

3. From the position of the pivotal plane we calculate radii of curvature (R_p) using Eq. 7. For each mixture at equilibrium in excess water (determined from the phase diagrams) we determine the spontaneous radius of monolayer curvature (R_{0p}).

4. The relation between the spontaneous radius of curvature, and the molar fraction of gramicidin, m_{gram}, can give an apparent spontaneous radius of curvature for each of the components if

$$\frac{1}{R_{0p}} = (1 - m_{gram}) \frac{1}{R_{0p}^{DOPE}} + m_{gram} \left(\frac{1}{R_{0p}^{gram}} \right) \qquad (9)$$

is linear.

5. The elastic energy given by Eq. 5 can be related to the osmotic work done by the osmotic stress (Π):

$$\Pi R_p^2 = 2K_{cp} \left(\frac{1}{R_p} - \frac{1}{R_{0p}} \right). \qquad (10)$$

A plot of (ΠR_p^2) versus ($1/R_p$) gives, from the slope and intercept, the monolayer bending modulus (K_{cp}) (Gruner et al., 1986)(Rand et al., 1990) and the radius of spontaneous curvature.

Temperature coefficients

Lattice dimensions were measured at 22°C, 30°C, and 40°C, where the sample was allowed to equilibrate at each temperature for at least 15 min before x-ray exposure. The temperature dependence, α_s, of the lattice dimension, s, ($s = 2d_{hex}/\sqrt{3}$) (Fig. 1) is described by the following (Rand and Pangborn, 1973):

$$\ln s = \alpha_s T + constant \qquad (11)$$

where the slope of the $\ln s$ vs. T plot yields the temperature coefficient α_s.

RESULTS

Structural characterizations for mixtures of gramicidin and DOPE

DOPE spontaneously forms the H_{II} phase when hydrated and at a dimension very close to its intrinsic curvature (Chen and Rand, 1998). The lattice dimensions of H_{II} phases formed by fully hydrated mixtures of gramicidin and DOPE, Fig. 2 A, show that d_{hex} decreases systematically as gramicidin increases up to mole fraction ~0.05, above which the dimensions change very little. The smaller changes may be due either to the exclusion of gramicidin from the H_{II} phase or to different interactions between gramicidin and DOPE that do not affect the H_{II} lattice dimensions.

From this experiment, it was concluded that the phases formed by mixtures of gramicidin and DOPE could be further analyzed for mole fractions up to 0.04 gramicidin.

Temperature coefficients of the H_{II} lattice dimension

The temperature coefficient α_s was measured in an attempt to determine whether gramicidin continued to be incorporated into the H_{II} phase above a mole fraction of 0.05. As seen in Fig. 2 B, α_s becomes less negative with increasing mole fraction gramicidin up to ~0.07. Above this composition, α_s does not change significantly ($p < 0.05$), suggesting that gramicidin is not incorporated beyond 0.07 mole fraction.

Phase diagrams for mixtures of gramicidin and DOPE

The H_{II} and L_α lattice dimensions of phases formed by mixtures of gramicidin and DOPE were measured over a range of water content (Fig. 3). The lattice dimensions for all compositions measured increased as the weight fraction of water to lipid ($1 - c$) increased until a maximum spacing was reached at full hydration. The dehydrated data for all gramicidin contents were pooled and empirically fit with a common exponential curve. The maximum repeat spacings at full hydration were averaged and fitted with a horizontal line. As the content of gramicidin increased up to mole fraction 0.04, the maximum repeat spacings systematically decreased, as listed in Table 1 and consistent with Fig. 2 A. The minimal water contents required for each phase to achieve full hydration, as determined from the intersection point of the exponential curve and the horizontal line, is referred to as equilibrium hydration, and are also listed in Table 1. In the dehydrated regions of the phase diagrams, all compositions of gramicidin exhibited the appearance and disappearance of an L_α phase, previously described for pure DOPE as the reentrant H_{II}-L_α-H_{II} transition (Gawrisch et al., 1992; Kozlov et al., 1994). These two-phase samples were

TABLE 1 The equilibrium water fraction $1 - c$ required to achieve the maximum lattice dimensions, and d_{hex} at full hydration, as determined from the phase diagrams (Fig. 3) for various contents of gramicidin in mixtures with DOPE

Mole fraction of gramicidin	Equilibrium water fraction $1 - c$	Maximum lattice dimensions d_{hex} (Å)
0.00	0.32	64.7
0.01	0.32	64.3
0.02	0.31	62.9
0.03	0.29	60.6
0.04	0.28	59.1

not used for further analysis because the composition of each phase was unknown. However, several dehydrated samples formed the H_{II} phase only, and were used in the diagnostic analysis described below.

Structural parameters for mixtures of gramicidin and DOPE

Structural dimensions within the fully hydrated H_{II} phase, shown in Fig. 1, have been plotted as they relate to gramicidin content in Fig. 4. The thickness of an effective bilayer (d_l) and the thickness of the hydrocarbon region of an effective bilayer (d_{lhc}) in the interaxial direction of the H_{II} phase both remain constant as the composition of gramicidin increases. The area of an effective molecule at the Luzzati plane (A_w) remains constant over the range of gramicidin composition. However, the areas of an effective molecule at the pivotal plane (A_p) and at the acyl chain terminals (A_t) both increase with increasing gramicidin content. These data will be used to discuss the structural effects of gramicidin on the H_{II} phase.

Diagnostic plot for mixtures of gramicidin and DOPE

Structural parameters were determined for the single hexagonal phases formed by DOPE and gramicidin and
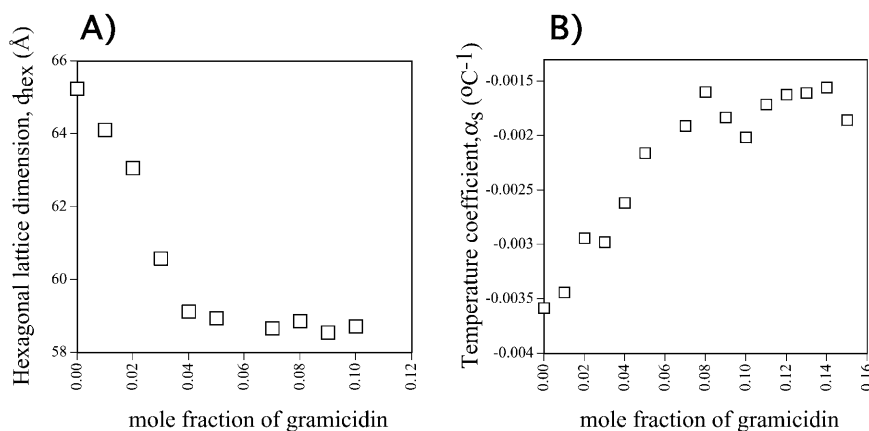
FIGURE 2 (*A*) The hexagonal lattice dimension d_{hex} of the fully hydrated H_{II} phases as they relate to the mole fraction of gramicidin in mixtures with DOPE. (*B*) The temperature dependence of the interaxial spacing s (see Fig. 1), of the H_{II} phase as it relates to the mole fraction of gramicidin in mixtures with DOPE. The temperature coefficient, α_s, for each composition was measured over a range of 20°C to 40°C.
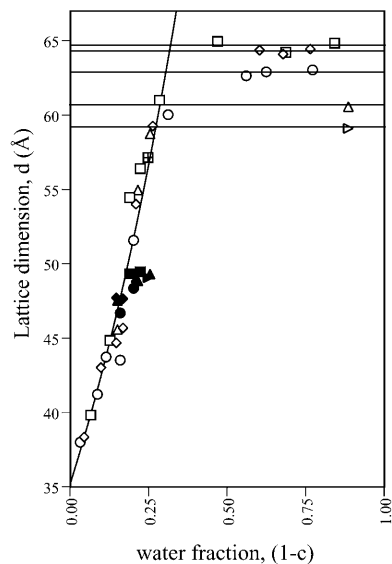
FIGURE 3   The lattice dimensions, d, as they relate to the amount of water added to samples containing various mole fractions of gramicidin in mixtures with DOPE. Symbols are (□) 0.00, (◇) 0.01, (○) 0.02, (△) 0.03, and (▷) 0.04 mole fractions. Closed symbols are lamellar phases and open symbols are hexagonal phases. With increasing water fractions but still dehydrated, d_{hex} increases until a maximum lattice dimension is reached at full hydration. The dehydrated data for all gramicidin contents were pooled and fitted with a common exponential curve. The maximum repeat spacings at full hydration were averaged and fitted with a horizontal line. Water fraction $(1 - c)$ and d_{hex} at the intersection points are listed in Table 1.

used to construct a diagnostic plot (Eq. 8, Fig. 5 *A*). All mole fractions of gramicidin (0.00, 0.01, 0.02, 0.03, and 0.04) resulted in a common, linear plot. This indicates that a pivotal
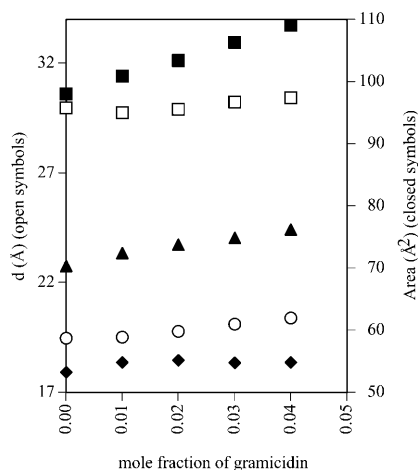

FIGURE 4   Through the geometrical relationships described in the Structural Analysis section, dimensions of the structural parameters shown in Fig. 1 are plotted as they relate to the mole fraction of gramicidin in mixtures with DOPE at equilibrium hydration. d_l (□) is the thickness of an effective bilayer in the interaxial direction, and d_{lhc} (○) is the thickness of the hydrocarbon region of an effective bilayer in the interaxial direction. The areas of an effective molecule are plotted at the Luzzati plane, A_w (◆), the pivotal plane, A_p (▲), and at the acyl chain terminals, A_t (■).

plane is well defined and exists at the same relative position, $V_p/V_l = 0.36$, for all gramicidin compositions examined. All radii of curvature are measured to this plane.

Spontaneous curvature of monolayers containing gramicidin and DOPE

The radii of spontaneous curvature (R_{0p}) for each gramicidin / DOPE mixture were determined from the fully hydrated structural parameters and the position of the pivotal plane according to Eq. 6. As the gramicidin content increased from mole fractions of 0.00–0.04, the spontaneous curvatures (1/R_{0p}) increased linearly (Fig. 5 *B*). This relationship can be described in a least squares linear fit as $1/R_{0p} = 0.107 m_{gram} + 0.033$. From this equation, the intrinsic curvature of gramicidin itself, $1/R_{0p}^{gram}$, is -0.14 Å^{-1}, and its radius of intrinsic curvature, R_{0p}^{gram}, is -7.1 Å. The intrinsic curvature of DOPE ($1/R_{0p}^{DOPE}$) is -0.033 Å^{-1}, with a corresponding radius of intrinsic curvature (R_{0p}^{DOPE}) of -30.3 Å, in agreement with several previously measured values (Chen and Rand, 1997; Epand et al., 1996; Leikin et al., 1996; Rand et al., 1990).

Osmotic stress exerted on mixtures of gramicidin and DOPE

Osmotic pressure exerted by PEG solutions on mixtures of gramicidin and DOPE alter the H_{II} lattice dimensions. For mole fractions of 0.01, 0.02, and 0.03 gramicidin, there was a coexistence of L_α and H_{II} phases at high osmotic pressures (Π), consistent with the H_{II}-L_α-H_{II} reentrant transition seen in Fig. 3. However, single H_{II} phases exist at lower osmotic pressures. For all compositions of gramicidin examined, the H_{II} lattice dimensions decreased as the osmotic pressure increased. This data was used to determine the bending moduli for mole fractions of 0.00, 0.01, 0.02, 0.03, and 0.04 gramicidin in DOPE as described by Eq.10 and shown in Fig. 6. These bending moduli ranged from 11 kT to 13 kT with no observable trend with gramicidin content.

Gramicidin in DOPC

The phases, and their dimensions, formed by mixtures of gramicidin in DOPC at full hydration are shown in Fig. 7. Most samples were prepared with tetradecane to reduce interstitial stress and allow the H_{II} phase to realize the lattice dimension of lowest free energy. Sjolund et al (Sjolund et al., 1989) have shown that alkanes alone can induce the hexagonal phase in certain conditions. In this system DOPC/td did not produce the hexagonal phase up to 60°C and so we take the transition we observe to be primarily due to gramicidin. Samples were also prepared without td to compare with other previous studies (Chupin et al., 1987; Killian et al., 1987; Killian and de Kruijff, 1985a,b; Killian et al., 1989, 1986; Van Echteld et al., 1982, 1981).
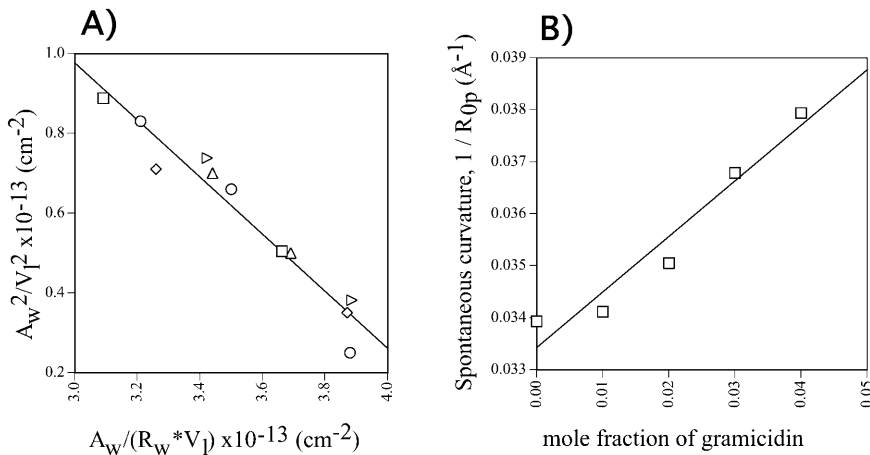
FIGURE 5 (A) The diagnostic plot (Eq. 8) for various gramicidin/DOPE mixtures. Symbols are (□) 0.00, (◇) 0.01, (○) 0.02, (△) 0.03, and (▷) 0.04 mole fraction gramicidin. All compositions of gramicidin yield a common, linear relationship indicating that there is a well-defined, common pivotal plane at a position (V_p/V_l) of 0.36. (B) Spontaneous curvatures of fully hydrated mixtures of gramicidin and DOPE, measured to the pivotal plane as a function of gramicidin content.

The results indicate that td has a qualitative and quantitative effect on the phase that forms by the mixture of DOPC and gramicidin. Without td the L_α phase persists and its lattice dimension increases up to mole fraction 0.04 gramicidin, at which point an H_{II} phase appears with a very small dimension. With td however, very little gramicidin is required to induce an H_{II} phase of large dimension that coexists with the L_α. At mole fractions of 0.03 gramicidin, the H_{II} phase transition is complete and the lattice dimension dramatically decreases with increasing gramicidin content. At mole fractions greater than 0.15 gramicidin the lattice dimension appears to change very little. It was concluded that gramicidin / DOPC mixtures could be studied, in the presence of td, between mole fractions of 0.07 and 0.14.

Phase diagrams for mixtures of gramicidin and DOPC

The hexagonal lattice dimension d_{hex} was determined as it varies with water content (Fig. 8). This was done for mole fractions of 0.07, 0.08, 0.10, 0.12, and 0.14 gramicidin in DOPC. Structural parameters were determined as for DOPE/gramicidin and are given in Table 2 for each content of gramicidin.

In most previous studies (Chen and Rand, 1997; Fuller and Rand, 2001; Leikin et al., 1996; Szule et al., 2002), and in the DOPE system of the present study, the hexagonal lattice dimensions in the dehydrated regions were common for all compositions. This shows that the dehydrated lattice dimensions are not dependent on the amount of inclusion but only on the fraction of water present. However in contrast to
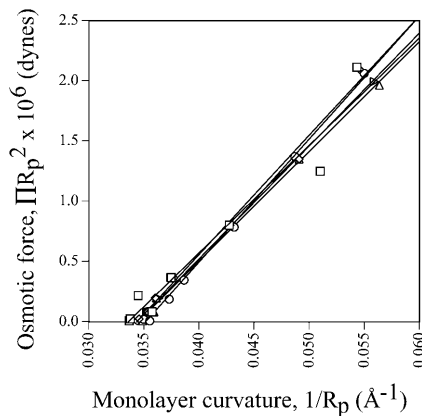

FIGURE 6 The osmotic force exerted by PEG 20,000 as it relates to monolayer curvature of the H_{II} phase measured to the pivotal plane (Eq. 10). These plots are derived for mole fractions of (□) 0.00, (◇) 0.01, (○) 0.02, (△) 0.03, and (▷) 0.04 gramicidin in mixtures with DOPE. The bending moduli for these compositions were determined from the slopes of each.
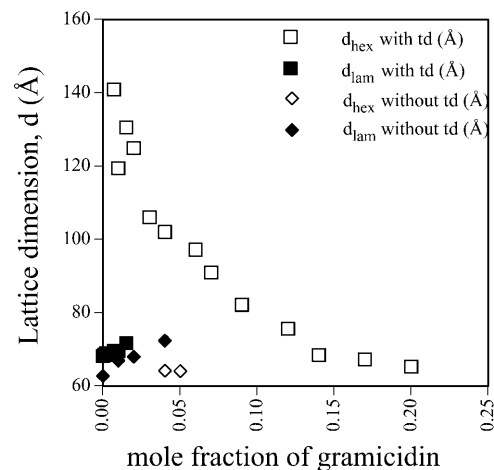

FIGURE 7 The lattice dimensions, d, of the reverse hexagonal phase (open symbols) and the lamellar phase (closed symbols) in the presence and absence of td as they relate to mole fraction of gramicidin in fully hydrated mixtures with DOPC.

TABLE 2 The equilibrium water fraction, $1 - c$, required to achieve the maximum lattice dimensions, d_{hex}, at full hydration, as determined from the intersection points on the phase diagrams (Fig. 8) for various compositions of gramicidin in mixtures with DOPC and td

Mole fraction of gramicidin	Equilibrium water fraction $1 - c$	Maximum lattice dimensions d_{hex} (Å)
0.07	0.41	83
0.08	0.40	81
0.10	0.38	72
0.12	0.33	69
0.14	0.30	65

this, various mixtures of gramicidin in DOPC do not yield common or systematically changing dehydrated dimensions (Fig. 8). Nevertheless these data can be used to determine several structural parameters (Fig. 9) and to construct a diagnostic plot (Eq. 8).

Structural parameters for mixtures of gramicidin and DOPC

Some structural dimensions within the H_{II} phases, as shown in Fig. 1, have been plotted as they relate to the gramicidin content (Fig. 9). The thickness of an effective bilayer (d_l), and the thickness of the hydrophobic region of an effective bilayer (d_{lhc}) in the interaxial direction remain relatively

FIGURE 9 Through the geometrical relationships described in the Structural Analysis section, the dimensions of the structural parameters shown in Fig. 1 are plotted as they relate to the mole fraction of gramicidin in mixtures with DOPC in the presence of td at equilibrium hydration. d_l (□) is the thickness of an effective bilayer in the interaxial direction, and d_{lhc} (○) is the thickness of the hydrocarbon region of an effective bilayer in the interaxial direction. The molecular areas of an effective molecule are plotted at the Luzzati plane, A_w (◆), and at the acyl chain terminals, A_t (■).

constant as the mole fraction of gramicidin increases. As the composition of gramicidin increases, the area of an effective molecule at the Luzzati plane (A_w) increases slightly compared to the relatively large increase in area at the acyl chain terminals (A_t) (Fig. 9). These data will be used to discuss the structural effects of gramicidin on the H_{II} phase.

Diagnostic plot of gramicidin in mixtures with DOPC

Different mole fractions of gramicidin in mixtures with DOPC did not yield a common plot (Fig. 10). Furthermore, the nonlinearity of these diagnostic plots for 0.07, 0.08, 0.10, and 0.14 mole fractions of gramicidin indicate that a well-defined pivotal plane does not exist for these compositions. On the other hand, 0.12 mole fraction of gramicidin does show a linear relationship which suggests the existence of a well-defined pivotal plane for this composition. If we accept this linearity as reflecting a true pivotal plane for this one composition, its position, (V_p/V_l), is 1.2. This ratio, greater than 1, means that the volume up to the pivotal plane (V_p) is greater than the volume of the effective molecule itself (V_l). By this, the pivotal plane exists outside the effective molecule, beyond the acyl chain terminals.

Spontaneous curvature of monolayers containing gramicidin and DOPC

From Eq. 9 and the calculated V_p/V_l, the spontaneous curvature ($1/R_{0p}$) of monolayers made of 0.12 mole fraction of gramicidin in DOPC is determined to be -0.022 Å^{-1}.
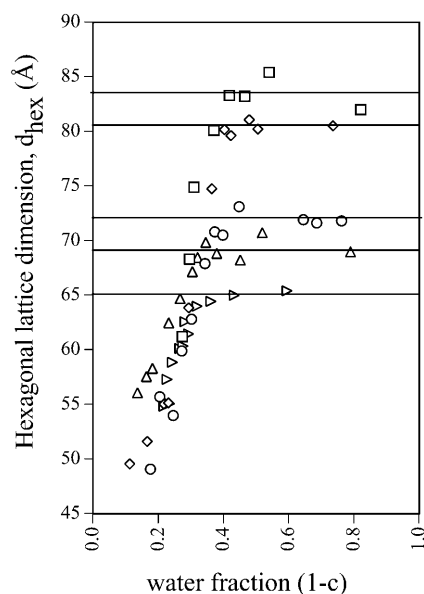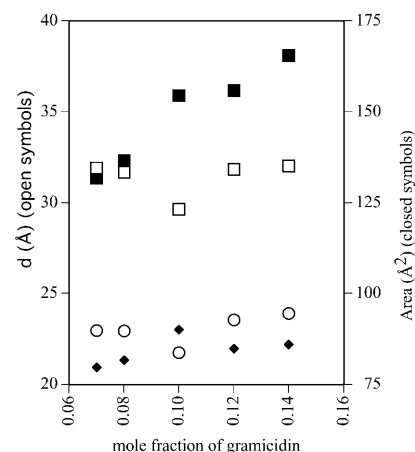

FIGURE 8 The hexagonal lattice dimension, d_{hex}, as it relates to the amount of water added to mole fractions of (□) 0.07, (◇) 0.08, (○) 0.10, (△) 0.12, and (▷) 0.14 gramicidin in mixtures with DOPC and td. With increasing hydration, but still limited water, d_{hex} increases until an equilibrium hydration spacing is reached at full hydration. The average d_{hex} value of each fully hydrated sample was fitted with a horizontal line for each composition. Water fraction ($1 - c$) and d_{hex} at the intersection points (described in the text) are listed in Table 2 for each composition.

FIGURE 10   Diagnostic plots for mole fractions of (□) 0.07, (◇) 0.08, (○) 0.10, (△) 0.12, and (▷) 0.14 gramicidin in mixtures with DOPC in the presence of tetradecane. The nonlinearity of the 0.07, 0.08, 0.10, and 0.14 compositions indicate that a pivotal plane does not exist. The linear relationship of the plot for the 0.12 composition indicates that a well-defined pivotal plane does exist at a position (V_p/V_l) of 1.2.
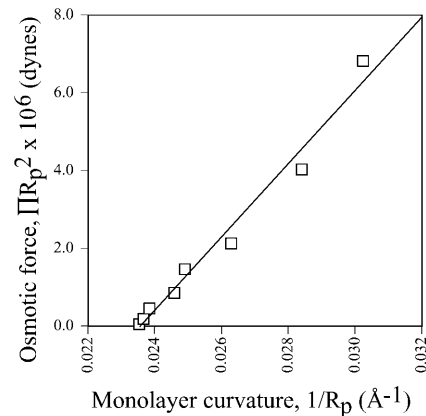

FIGURE 11   The osmotic force as it relates to monolayer curvature of H_{II} phases measured to the pivotal plane (Eq. 10). This data is derived for the system composed of 0.12 mole fraction of gramicidin in mixtures with DOPC and td. The bending modulus, K_{cp}, could be determined to be 115 kT from the slope of this linear relationship.

Linear addition of component curvatures cannot be demonstrated in these mixtures. However its assumption can lead to an estimate of the curvature of gramicidin itself. The intrinsic curvature of DOPC determined from a previous study is -0.0066 Å^{-1} (Szule et al., 2002). Using this, the intrinsic curvature of gramicidin itself $(1/R_{0p}^{gram})$, when mixed to 0.12 mole fraction in DOPC is determined to be -0.13 Å^{-1} making R_{0p}^{gram} equal to -7.4 Å. In spite of this limited data and tenuous assumption of additive curvatures, this result is remarkably similar to that measured for gramicidin in mixtures of DOPE where linear addition of curvatures was shown to apply.

Osmotic stress on mixtures of gramicidin and DOPC

The lattice dimensions of the osmotically equilibrated samples showed the same anomalies and inconsistencies as they did in the phase diagrams. This all points to a degree of complexity of interactions between DOPC and gramicidin that precludes easy analysis.

However, accepting that a well-defined pivotal plane appears to exist at a mole fraction of 0.12 gramicidin would allow an estimate of a bending modulus to be made for this composition (Fig. 11). A value of 115 kT suggests very stiff monolayers. Based upon the cross-sectional dimensions of gramicidin, (~15 Å (Wallace, 1986)) and the lipids, (3.9 Å (Chen and Rand, 1998)), it is estimated that ~8–9 lipids are required to surround a single gramicidin molecule. Contact between gramicidin molecules might therefore begin at ~0.09–0.10 mole fraction, possibly leading to the observed monolayer stiffness.

DISCUSSION

We have attempted to measure for the first time the curvature parameters of a peptide inclusion in a phospholipid monolayer. Such parameters have been measured successfully for a range of more natural lipidic inclusions such as various phospholipid species, cholesterol and alpha tocopherol (Leikin et al., 1996; Fuller and Rand, 2001; Chen and Rand, 1997; Bradford, 2000). The peptide gramicidin was selected for its hydrophobic properties and the comprehensive characterization of its gating properties and how they are affected by its lipid host. Our attempts have been met with mixed success. In DOPE monolayers in the hexagonal phase, gramicidin is well behaved. In DOPC, it is not well behaved. Gramicidin has a variety of environment-dependent conformations that have a long history of controversy (reviewed by Ramachandran, 1975; Wallace, 1986; Killian, 1992; Andersen et al., 1996). Their conformation in the hexagonal phase is not known, and we can only treat it as an inclusion whose effects on dimensions we can accurately and systematically measure. In the case of DOPE, but not DOPC, we have determined a range of compositions over which gramicidin behaves uniformly and we proceed with analyzing the structural changes as we have for other inclusions. In the case of DOPC, the behavior is not uniform and our conclusions are limited.

Mixtures of gramicidin and DOPE

Between 0 and 0.04 mole fraction gramicidin, there is a systematic reduction of H_{II} lattice dimension from that of pure DOPE, indicating that increased negative curvature is introduced by gramicidin. For compositions greater than 0.04 mole fraction gramicidin, d_{hex} is small and remains constant, suggesting that gramicidin's curvature contribution

is somehow altered or the peptide is excluded from the H_{II} phase. Our interpretation of the temperature coefficients seen in Fig. 2 B is that gramicidin is not incorporated beyond mole fraction 0.06–0.07 in DOPE.

The different changes in molecular area along the length of the DOPE molecule (Fig. 4) show that gramicidin is contributing molecular area and volume deep in the hydrophobic regions of the monolayer and not at the polar group/water interface. This is consistent with the models of gramicidin gating, bound deep in the monolayer and sensitive to bilayer thickness. It is also consistent with the proposal that inter-PE hydrogen bonds interfere with interactions between the tryptophan residues of gramicidin and the polar regions (Scarlata and Gruner, 1997). Consequently, gramicidin would be more restricted to the hydrophobic regions of these monolayers.

The radius of intrinsic curvature for gramicidin measured in mixtures with DOPE (R_{0p}^{gram}) is −7.1 Å. This value should not be interpreted in terms of the effective shape of a molecule as is done for the lipids. Rather the value reflects that gramicidin behaves as if it contributes high negative curvature because it partitions largely into the hydrophobic part of the monolayer. Its effect is large considering that diacylglycerols and cholesterol, both powerful H_{II}-phase inducing lipids, introduce less negative curvature into a membrane than does gramicidin (Szule et al., 2002; Leikin et al., 1996; Chen and Rand, 1997).

Gramicidin could not be observed to affect the bending modulus of DOPE monolayers. This could be due to the relatively small mole ratios of peptide to lipid, where the local effects of gramicidin may be too diluted to be observed. At these compositions, there are more than enough lipids to completely surround each gramicidin molecule, and our measurements may not reflect the local bending modulus around a gramicidin molecule. The very high bending modulus tenuously estimated for gramicidin in DOPC may better reflect that local bending, because the relative amount of lipid is less.

Mixtures of gramicidin and DOPC

Gramicidin in DOPC induces the lamellar-hexagonal phase transition indicating a large contribution of negative curvature. Typically, without td, the H_{II} phase of gramicidin and DOPC mixtures have a small and invariant lattice dimension. Such small and invariant lattice dimensions have been observed for other mixtures of gramicidin and different phospholipids, (Chupin et al., 1987; Killian et al., 1987; Killian and de Kruijff, 1985a,b, Killian et al., 1989, 1986; Van Echteld et al., 1982, 1981) and are likely a result of chain stress, i.e., a requirement for the interstices to be filled. Such systems cannot reflect the spontaneous curvature of these mixtures. For curvature studies on systems of low spontaneous curvature, td is required to fill the interstices, allowing the monolayers to form the curvature of lowest free energy

(Gruner, 1985). Our practice is to include td, allowing the small curvatures and large dimensions to be expressed. Under these conditions the L_α to H_{II} phase transition is complete with as little as ~0.03 mole fraction gramicidin in DOPC.

The structural parameters calculated and shown in Fig. 9 indicate that gramicidin in DOPC, as in DOPE, is mainly contributing area and volume to the hydrophobic region of an effective molecule, thereby adding negative curvature to the monolayers.

From the diagnostic plots, it is clear that a well-defined pivotal plane does not exist at most compositions. This is the first inclusion for which we have observed this. Nevertheless a well-defined pivotal plane appears to exist at 0.12 mole fraction gramicidin in DOPC. This composition is where each gramicidin can be surrounded with only one layer of lipid, were it uniformly distributed in the monolayers, and this may restrict structural rearrangements on dehydration. On this tenuous basis we proceeded with the curvature analysis which yielded a position for the pivotal plane at V_p/V_l greater than 1. This means that the monolayers are bending around a plane outside of the lipid molecules, beyond the acyl chain terminals. At all positions along the entire length of the effective molecule the area is changing in the same direction as the monolayers bend. This is qualitatively different from the bending of any other monolayer we have observed.

One can obtain an estimate of the intrinsic curvature of gramicidin itself in this mixture. This requires a), the intrinsic curvature of DOPC itself, which was determined from a previous study (Szule et al., 2002) and b), the unproved assumption that DOPC and gramicidin curvatures add linearly. On this basis gramicidin molecules are behaving as though they have an intrinsic radius of curvature, R_{0p}^{gram}, of −7.4 Å in DOPC. Remarkably, in spite of these severe assumptions, this value is very close to that obtained in the DOPE system, 7.1 Å. This suggests that gramicidin is contributing the same relative hydrophobic volume to the effective molecules in each lipid system.

A bending modulus, K_{cp} could only be measured for this composition. A K_{cp} of 115 kT indicates that these monolayers are very stiff. They are an order of magnitude more resistant to change in curvature than monolayers composed of gramicidin and DOPE, and any other lipid system we have measured. Such stiffness may be a result of the high concentration of gramicidin found within the monolayers, higher than could be achieved with DOPE, and at a level where only one lipid is available to separate gramicidins. This may reflect bending moduli local to gramicidin more realistically. Lateral interactions between adjacent peptides, perhaps through interactions of tryptophans, may contribute to this observed stiffness (Killian and de Kruijff, 1986). Also, interaxial hydrogen bonds of single-stranded dimers may provide the phase structure with a more rigid backbone, as proposed by Van Echteld et al. (1981).

Why are the interactions of gramicidin with DOPE different from DOPC? PEs are able to form intermolecular hydrogen bonds, thereby reducing the effective size and hydration of the polar region. They also prevent hydrogen bonding between the tryptophan residues and the polar regions of the lipids (Scarlata and Gruner, 1997). It was postulated that gramicidin is excluded from this polar group, residing deeper in the hydrocarbon region. PCs on the other hand, do not form strong inter-headgroup associations, are more fully hydrated and allow hydrogen bonds between the tryptophan residues and the polar region (Scarlata and Gruner, 1997). This would position gramicidin closer to the polar groups of DOPC than DOPE. Such a looser polar group region in DOPC may allow the position of gramicidin in DOPC monolayers to change with dehydration and bending. Something like this may account for the variation in dimensions seen in the DOPC system.

Given these qualitative differences between PC and PE, one might expect to observe qualitative differences in the gating properties of gramicidin in these two different membranes. In PC/PE mixtures it is found that the channel lifetime decreases as the mole fraction of PE increases (Maer, A. M., J. A. Lundbæk and O. S. Andersen, personal communication). This provides hope of eventually connecting gating properties with such structural parameters as determined in this study.

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