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Orientation of gramicidin D incorporated into phospholipid multibilayers: a Fourier transform infrared-attenuated total reflection spectroscopic study

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Polarized Fourier transform infrared (FTIR)–attenuated total reflection (ATR) spectroscopy was applied to study the orientation of the linear pentadecapeptide antibiotic gramicidin D incorporated into phospholipid multibilayers, which were cast on a germanium ATR plate from chloroform solution. In DMPC and DPPC multibilayers, the CH_2 stretching bands of lipid hydrocarbon chains were slightly shifted to the higher frequency side and bandwidth was increased in the presence of gramicidin. However, in DPPE multibilayers, frequencies and bandwidths of these bands were unaltered. In each case, gramicidin produced little effect on the orientation of lipid hydrocarbon chains, suggesting that gramicidin penetrates into lipid layers without noticeable perturbations. Upon incubation of cast films in contact with water above the gel–liquid-crystalline transition temperature (T_c) of lipids, the reorientation of gramicidin in lipid multibilayers occurred, the degree thereof depending upon the fluidity of the lipid hydrocarbon chains and the amount of surrounding water. In DMPC multibilayers, the helix axis of gramicidin was oriented almost parallel to the lipid hydrocarbon chains after incubation. In DPPC multibilayers, on the other hand, the helix axis of gramicidin was tilted on average about 15° from the lipid hydrocarbon chains after incubation. However, in DPPE multibilayers, which are known to have the most rigid bilayer structures, the reorientation of gramicidin could not be seen.

Introduction

Gramicidin D isolated from *Bacillus brevis* is a linear pentadecapeptide antibiotic consisting of alternating hydrophobic L- and D-amino acids. It is known to form transmembrane channels in natural and artificial lipid bilayer membranes, facilitating passive transport of alkali metal ions [1,2]. So far, there have been many conformational studies of gramicidin D by means of CD [3–8], infrared [6,9–12], Raman [7,9,12], NMR [5,13–18] and X-ray [19] analyses. Most of these studies have shown that the transmembrane channel is composed of dimeric gramicidin mainly forming π_{LD} helices associated at the NH_2 terminals which are different from those in organic solvents. These were reviewed in detail by Higashijima and Miyazawa [20]. Furthermore, theoretical considerations of the gramicidin channel have recently been given in terms of energy profile [21] and by the atomic coordinate calculation of helices [22].

There have been considerable numbers of infrared studies on lipid-protein interactions with the aid of various techniques such as differential [23], Fourier transform [24,25], attenuated total reflection [26,27] and polarization [26–32] spectroscopies. From infrared dichroic measurements, Nabedryk et al. [30] have concluded that the gramicidin transmembrane channel in dimyristoylphosphatidylcholine (DMPC) vesicles is oriented

almost parallel to the hydrocarbon chains of DMPC.

In this paper, Fourier transform infrared (FTIR)–attenuated total reflection (ATR) spectroscopy was applied to study gramicidin-incorporated phospholipid multibilayers of DMPC, dipalmitoylphosphatidylcholine (DPPC), and dipalmitoylphosphatidylethanolamine (DPPE). Infrared dichroic measurements were carried out to evaluate the orientation of gramicidin D with respect to that of the hydrocarbon chains of lipids. Attention was paid to the effect of rigidity of multibilayer-forming lipids and the presence of surrounding water (incubation) upon orientation of gramicidin D in lipid multibilayers.

Materials and Methods

Gramicidin D (Dubos) and phospholipids (DMPC, DPPC and DPPE) were purchased from Sigma and used without further purification. Chloroform used as a solvent was a specially prepared reagent (UVS-26) from Nakarai Chemicals Co. Ltd., Japan. Water was purified by a Mitamura Riken model PLS-DFR automatic lab-still consisting of a reverse-osmosis module, an ion-exchange column, and a double distiller.

A stock solution of 3 mg/ml was prepared by dissolving gramicidin D and each phospholipid in chloroform at a ratio of 8–10 lipids per gramicidin. After spreading 300–400 μl of the stock solution on one face of a germanium ATR plate ($52 \times 18 \times 2$ mm), the solvent was carefully evaporated to form a dry well-oriented film about 1 μm thick. The film thus prepared was overlaid with water and incubated at a temperature above the gel–liquid-crystalline transition temperature (T_c) of each lipid; 30°C for DMPC ($T_c = 24$°C), 50°C for DPPC ($T_c = 42$°C), and 70°C for DPPE ($T_c = 63$°C). After air-drying at room temperature, the sample was subjected to polarized infrared ATR measurements.

Polarized spectra were recorded on a Nicolet model 6000C FTIR spectrophotometer equipped with an MCT detector and a wire grid polarizer. Regarding the ATR measurements, the angle of incidence was 45° and the number of reflections was 12 on the sample side and 25 as a whole. 500 interferograms were accumulated to yield spectra of high S/N ratio with resolution of 4 cm^{-1}. The frequency reading was accurate to within ± 0.1 cm^{-1}.

Results

Infrared ATR spectra of gramicidin-containing phospholipid multibilayers

Fig. 1 shows the representative polarized infrared ATR spectra of gramicidin-containing DMPC multibilayers. In this figure, (a) and (b) refer to the sample before and after incubation above T_c, respectively. Frequencies of the antisymmetric and symmetric CH_2 stretching and the ester C=O stretching bands of DMPC multibilayer in the presence and the absence of gramicidin D are shown in Table I, together with those for DPPC and DPPE multibilayers. Frequencies of the amide I band (the C=O stretching vibration) of gramicidin D incorporated into various lipid multibilayers are shown in Table II. All of these values are the averages for several samples and are in good agreement with those reported in previous papers [30,33–35]. Besides those, there are the gramicidin bands at approx. 3280 and approx. 1550 cm^{-1} assignable to the amide A band (the NH stretching vibration) and the amide II band (the coupled mode of the CN stretching and the NH bending vibrations) as well as the lipid band at 1468 cm^{-1} assignable to the CH_2 scissoring vibration in Fig. 1. Fortunately, there is no serious overlapping of the bands between gramicidin and lipids.

Fig. 1 also indicates typical dichroic features of each band. First, for DMPC bands such as the CH stretching bands of the alkyl chains at 3000–2800 cm^{-1} and the ester C=O stretching band at approx. 1740 cm^{-1}, no appreciable changes in dichroism were observed upon incubation. On the other hand, for gramicidin bands such as the amide A band at approx. 3280 cm^{-1} and the amide I band at approx. 1640 cm^{-1}, the parallel dichroism was largely augmented upon incubation.

Effect of gramicidin D on the CH_2 stretching bands of phospholipid multibilayers

With regard to the effect of gramicidin D on the lipid bands, it is seen from Table I that, in DMPC and DPPC multibilayers, the antisymmet-

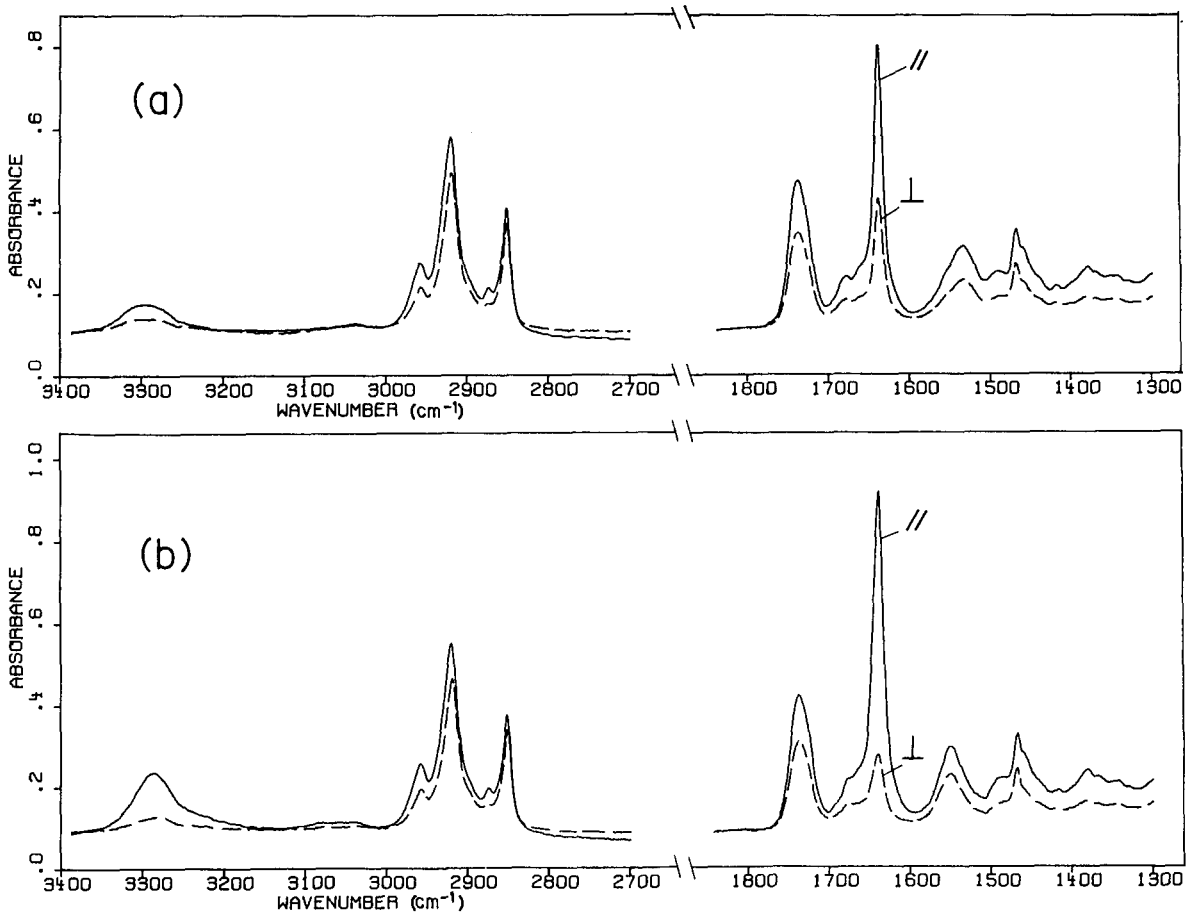

Fig. 1. Polarized infrared ATR spectra of the DMPC-gramicidin D system at a ratio of 10 lipids per gramicidin. (a) before and (b) after incubation above T_c. Solid and broken lines refer to the direction of electric vector parallel and perpendicular to the plane of incidence, respectively.

ric CH_2 stretching bands slightly shift to the higher frequency side in the presence of gramicidin D. Moreover, the bands are broadened by the introduction of gramicidin D. In DPPE multibilayers, however, there appears to be little effect of gramicidin D on the frequency and bandwidth of

TABLE I

EFFECT OF GRAMICIDIN D ON THE SPECTRAL PARAMETERS OF LIPID BANDS

	Wavenumber (cm^{-1})			Halfbandwidth (cm^{-1})	Dichroic ratio	
	antisym. CH_2 str.	sym. CH_2 str.	C=O str.	antisym. CH_2 str.	antisym. CH_2 str.	sym. CH_2 str.
DMPC	2918.1±0.1	2850.4±0.1	1736.9±0.3	16.3±0.4	1.0±0.1	1.0±0.0
DMPC+GD	2919.4±0.2	2851.0±0.1	1737.3±0.4	19.0±0.1	1.2±0.1	1.1±0.1
DPPC	2917.6±0.1	2850.2±0.2	1736.9±0.2	14.7±0.1	1.1±0.0	1.0±0.1
DPPC+GD	2918.9±0.1	2850.7±0.1	1737.3±0.3	17.3±0.2	1.2+0.1	1.1±0.1
DPPE	2917.5±0.2	2850.0±0.1	1738.6±0.2	15.1±0.4	1.0±0.0	1.0±0.1
DPPE+GD	2917.7±0.1	2850.4±0.2	1739.3±0.5	15.2±0.5	1.1±0.1	1.1±0.0

TABLE II

FREQUENCIES OF THE AMIDE I BAND AND CHANGE IN THE ORIENTATION OF GRAMICIDIN D BEFORE AND AFTER INCUBATION ABOVE T_c

	Wavenumber (cm^{-1})	Before incubation		After incubation	
		r [a]	α [b]	r [a]	α [b]
Pure GD film	1635.0±0.2	1.2±0.1	73±4°	1.3±0.2	70±5° [c]
GD in DMPC	1639.4±0.2	2.1±0.2	53±3°	4.8±0.3	32±2°
GD in DPPC	1638.4±0.5	1.6±0.2	62±5°	2.8±0.2	45±2°
GD in DPPE	1636.3±0.2	1.6±0.1	62±2°	1.8±0.1	58±2°

[a] Dichroic ratio of the amide I band of gramicidin.
[b] Average tilting angle of the helix axis of gramicidin with respect to the surface normal.
[c] For a pure gramicidin film, the temperature of incubation is 70°C.

the antisymmetric CH_2 stretching band.

The dichroic ratio defined by

$$r = \Delta A_{\parallel} / \Delta A_{\perp} \quad (1)$$

was calculated from the polarized ATR spectra. Here ΔA is the change in reflection absorbance due to the presence of a film. In Table I the values of dichroic ratio are also shown for lipid antisymmetric and symmetric CH_2 stretching bands. It is seen that gramicidin D has little effect on the dichroic ratio of lipid hydrocarbon bands, irrespective of the sort of multibilayer-forming lipids. Since the dichroism is related to the molecular orientation, this fact indicates that the orientation of lipid hydrocarbon chain is not appreciably changed by the introduction of gramicidin D, as described later in detail.

Effect of phospholipid on the gramicidin amide I band

Fig. 2 shows the infrared ATR spectra in the amide I band region of gramicidin D. There are no lipid bands in this region. The amide I band of the pure gramicidin film cast from chloroform has an asymmetric shape with an intense peak maximum at 1635 cm^{-1} and two shoulders at 1653 and 1685 cm^{-1} (d), being in good agreement with that reported in the previous paper [9]. However, the amide I band of gramicidin incorporated into phospholipid multibilayers is rather simple; the shoulders at 1653 and 1685 cm^{-1} diminish. Further, the peak maximum shifts to the higher frequency side, as seen in Fig. 2 and Table II. The amount of the frequency shift is largest for DMPC and decreased in the order DPPC > DPPE.

Change in the orientation of gramicidin D

As stated above, the dichroic ratio of the amide I band of gramiciding D is changed upon incubation in DMPC multibilayer. This is quantitatively shown in Table II. Also shown are the results for pure gramicidin D and that in DPPC and DPPE multibilayers. In a pure gramicidin film, the dichroic ratio is 1.2 before incubation and scarcely changed after incubation. However, the dichroic ratio of this band is found to be larger in lipid multibilayers; it is 2.1 in DMPC, and 1.6 in DPPC and DPPE before incubation. After incubation for

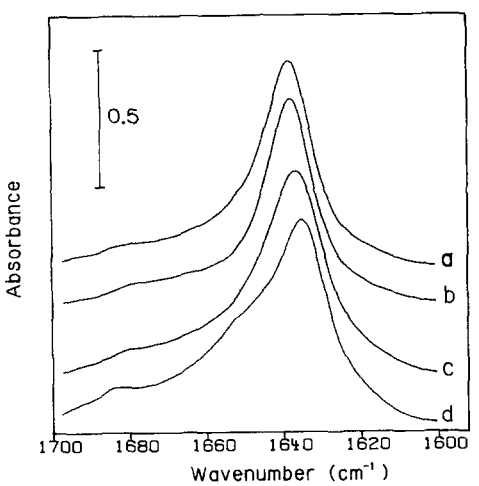

Fig. 2. The amide I band of gramicidin D in (a) DMPC, (b) DPPC, and (c) DPPE multibilayers. The curve (d) refers to the pure gramicidin D film cast from chloroform.

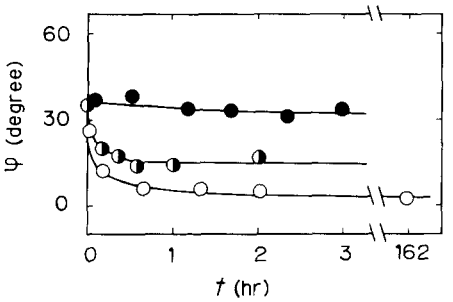

Fig. 3. Incubation time (t) dependence of the orientation of gramicidin D in DMPC (○), DPPC (◑), and DPPE (●) multibilayers.

1 h, these are increased by extents which depend upon the sort of multibilayer-forming lipids, i.e., the ratio is markedly increased from 2.1 to 4.8 in DMPC, and moderately increased from 1.6 to 2.8 in DPPC. The change in DPPE is within experimental error. It will be shown later that this dichroic ratio, and therefore the molecular orientation of gramicidin, is also subject to change by incubation time.

Discussion

Molecular interaction between lipids and gramicidin D

The introduction of gramicidin D into DMPC and DPPC multibilayers gives rise to a slight shift to higher frequency and broadening of the antisymmetric CH_2 stretching bands of the lipid hydrocarbon chains, remaining their dichroism unaltered (Table I). Since frequencies of CH_2 stretching bands depend upon the order of the lipid hydrocarbon chains and bandwidths depend upon the librational and torsional motion of the chains [23], these facts suggest that the introduction of gramicidin results in a reduction of the order of the hydrocarbon chains caused by the conformational change from the *trans*-zigzag form to the *gauche* form, as well as in the increase in the lipid hydrocarbon chain motion. Furthermore, since the dichroism of the lipid CH_2 stretching bands was unaltered in the presence of gramicidin D, gramicidin interacts only with the neighbouring lipid molecules, having no effects on the other parts of the lipid layers. The fact that gramicidin is incorporated into lipid bilayers without perturbation of lipid layers has also been suggested by DSC measurements [36–38]. Further, the same type of incorporation has been reported for other polypeptides such as proteolipid apoproteins [36,37] and synthetic lysine-leucine copolymers [39].

The present result seems contradictory to the previous finding in the polarized infrared studies of Nabedryk et al. [30], who have pointed out a slight reorganization in gramicidin-incorporated DMPC liposomes with a more perpendicular orientation of alkyl chains to the bilayer surface. However, this contradiction may be ascribed to the difference in the peptide/lipid ratio. Nabedryk et al. [30] adopted a peptide/lipid ratio of 1 : 30, which is much smaller than the present case (1 : 8–1 : 10). According to Lee et al. [23], low concentrations of gramicidin cause a small decrease in lipid chain *gauche* conformers as well as a slightly higher orientation, while high concentrations of gramicidin result in an increase in the number of lipid chain *gauche* conformers above the lipid phase transition temperature.

It has been reported that the aggregation of gramicidin molecules occurs at high concentrations [38,40]. In the present case, however, the aggregation of gramicidin is negligible, since the spectra in the amide I band region of incorporated gramicidin differ from the spectrum of pure gramicidin film, as shown in Fig. 2. The frequency shifts of the amide I band suggest the change in environments around the gramicidin molecules and/or its conformational change caused by the introduction of gramicidin into lipid multibilayers.

Molecular orientation of gramicidin D in phospholipid multibilayers

In order to calculate parameters of the molecular orientation of gramicidin D from the dichroic ratio (r) of the amide I bands, we applied Flournoy and Schaffers' equation [41] to the ATR spectra of a three-phase plane-bounded system with germanium (phase 1), a sample film (phase 2) and air (phase 3), because the sample film is thicker than the penetration depth. In this system we have Eqns. 2 and 3 for the infrared radiation with electric vectors perpendicular and parallel to the plane of incidence, respectively.

$$\Delta A_{\perp} = 0.301 k_x N \qquad (2)$$

$$\Delta A_{\parallel} = (0.256k_y + 0.345k_z)N \quad (3)$$

Here, k_x, k_y, and k_z are the absorption indices of the film along the x, y, and z axes. The x axis is perpendicular to the plane of incidence (the yz plane), the y axis is parallel to the germanium surface, and the z axis is normal to it. N is the number of total reflections at the sample-germanium interface. The coefficients in Eqns. 2 and 3 were obtained by using proper values of 4.00 and 1.44 for the refractive indices of germanium and film, respectively. In order to correlate the values of k_x, k_y, and k_z with the order parameter of gramicidin, the following model is assumed. First, the gramicidin molecule has a π_{LD} helical conformation associated at the NH_2 terminals as already proposed [3,4,20,30], and the helix axis of gramicidin is uniaxially oriented with an average angle of α with respect to the surface normal (z-axis). Second, the transition moments of the amide I band are uniformly distributed with the angle θ around each helix axis. According to Nabedryk et al. [30], the value of θ can be estimated to be 22.6°. Under this assumption the order parameter (F) of the helix axis of gramicidin with respect to the surface normal can be expressed by

$$F = \frac{k_z - k_x}{k_z + 2k_x} = \frac{3\cos^2\alpha - 1}{2} \cdot \frac{3\cos^2 22.6° - 1}{2} \quad (4)$$

Here, $k_x = k_y$ because of the uniaxial orientation around the z-axis. From Eqns. 1, 2, 3 and 4, the value of F is related to the dichroic ratio (r) of the amide I band as follows

$$F = \frac{r - 2.00}{r + 1.45} \quad (5)$$

The average tilting angles α of the helix axis of gramicidin calculated from observed r values are shown in Table II. Before incubation, gramicidin in DMPC gives a result of $\alpha = 53°$. This particular value means two types of orientation: one is the uniaxial orientation of the helix axis with an average angle of 53°, and the other is almost random orientation. This is because in the case of random orientation, $F = 0$ and therefore $\alpha = 54.7°$. Gramicidin in DPPC and DPPE, however, tends to have rather a parallel orientation to the film surface. After incubation, gramicidin in DMPC shows a tendency to reorient more vertically to the film surface. However, the helix axes of gramicidin in DPPC and DPPE multibilayers are still less vertical than those in DMPC.

The order parameters of lipid hydrocarbon chains can be calculated from the dichroic ratios shown in Table I with the aid of Eqn. 5. In this case, the transition moments of the antisymmetric and symmetric CH_2 stretching modes are assumed to be uniformly distributed with an angle of 90° ($\theta = 90°$) around each chain axis which is uniaxially oriented at an average angle ψ with respect to the surface normal. The order parameters were calculated to be -0.36 ± 0.07 in this case, and then the average tilting angles ψ of $25 \pm 7°$ were obtained irrespective of the sort of lipids. This value is similar to the previously reported ones [33,34,42], indicating that the films are well-oriented. Furthermore, this value is changed little not only by the introduction of gramicidin but also by incubation.

Returning to the orientation of gramicidin D in multibilayers, it was found to be subject to change by the incubation time t. Fig. 3 shows the time dependence of the molecular orientation of gramicidin in the form of the φ vs. t curve. Here, φ ($= \alpha - \psi$) is the average tilting angle of the helix axis of gramicidin with respect to the axis of lipid hydrocarbon chains. Therefore, $\varphi = 0°$ designates the parallel orientation of the helix axes along the lipid hydrocarbon chains. It is seen from Fig. 3 that the effect of incubation appears in a few minutes and the equilibrium φ values about 4° are obtained within 1 h for gramicidin in DMPC multibilayers, suggesting that gramicidin in this multibilayer is reoriented almost parallel to the lipid hydrocarbon chains in a short time. For gramicidin in DPPC multibilayer, on the other hand, the incubation effect also appears in a few minutes, but the helix axes of gramicidin are tilted on average about 15° from the lipid hydrocarbon chains after equilibrium has been achieved. In DPPE multibilayers, however, no appreciable effect of incubation is seen and a poor orientation of the helix axes is maintained during incubation for 3 h.

It is noteworthy that these orientational features of gramicidin are comparable to the change

in the spectral parameters of the lipid bands. The frequency shifts and the change in halfbandwidths of the lipid CH_2 stretching bands caused by the introduction of gramicidin occur in DMPC and DPPC. Correspondingly, the reorientation of gramicidin is observed by incubation of these multibilayers. However, neither spectral change by the incorporation of gramicidin nor the orientation of gramicidin by the incubation occurs in DPPE multibilayer. These facts reveal that the reorientation of gramicidin occurs by incubation in multibilayers where the order of the lipid hydrocarbon chains is reduced and librational and torsional motion of the chains is activated by the introduction of gramicidin. In other words, the flexibility and fluidity of the lipid hydrocarbon chains in multibilayers play an important role in the molecular orientation of gramicidin.

Furthermore, the surrounding water has a strong effect on the reorientation of gramicidin by incubation. In DMPC and DPPC multibilayers, the surrounding water easily penetrates into the polar part of the bilayers because of the weak electrostatic interaction between bulky head groups. The penetration of water results in looser packing of the lipid bilayers and consequently in increased flexibility of the lipid hydrocarbon chains. Therefore, the reorientation of gramicidin becomes feasible in these multibilayers, as in the case of the linear peptide antibiotic alamethicin, which facilitates ion transport [26,27]. In DPPE multibilayers, however, the surrounding water has difficulty in penetrating into the inner part of the bilayer, because of the strong electrostatic interaction between compact head groups. Therefore, no reorientation can be expected in this multibilayer.

It is therefore concluded that the fluidity of lipid layers and the surrounding water are essential for manifesting the function of gramicidin as a transmembrane channel.

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