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Effect of gramicidin addition upon the physicochemical properties of dipalmitoyl phosphatidyl choline large unilamellar vesicles

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Abstract

In this work, we report the results bearing on the effect of gramicidin incorporation upon the dynamic and structural properties of dipalmitoyl phosphatidyl choline (DPPC) large unilamellar vesicle (LUVs). Results were obtained at different depths in the bilayer, both in the bulk of the bilayer and the vicinity of the added pentadecapeptide. This analysis was performed employing a series of extrinsic fluorescent probes (1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), 6-dodecanoyl-2-dimethyl aminenaphthalene (Laurdan), pyrenedodecanoic acid, pyrene butyric acid, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (Py–C_{10}–PC) and 1,2-bis-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (bis-Py–C_{10}–PC)). Fluorescence measurements were carried out after direct excitation of the probe or after its excitation by resonant energy transfer (RET) from the intrinsic tryptophan groups of the polypeptide. The last procedure allows an evaluation of the lipidic annulus properties in the vicinity of the incorporated polypeptide.

In the gel state, gramicidin incorporation increases the order near the vesicle interface, as sensed by TMA-DPH. This increase is observed, above the phase transition temperature, over all the bilayer. This effect is probably related to a decrease in the lateral diffusion of the lipids, as evidenced by the reduced formation of intermolecular excimers following Py–C_{10}–PC excitation. Gramicidin incorporation also increases the penetration and/or mobility of water molecules located near the bilayer interface. In the gel phase, these changes are more relevant in the central part of the bilayer. When the LUVs are in the liquid crystalline state, the effect is observed through all the bilayer. In this state, the polypeptide incorporation also increases the diffusion/concentration of oxygen, irrespective of the probe localization.

Resonance energy transfer between Trp residues and Laurdan, Py–C_{10}–PC or bis-Py–C_{10}–PC dyes was employed to evaluate the properties of the lipidic annulus surrounding the polypeptide. In the gel phase, gramicidin incorporation produces an increase in the penetration and/or dynamics of water molecules, while in the liquid crystalline state it leads to a decrease of the acyl chain mobility in the deepest parts of the bilayer.

Keywords: Large unilamellar vesicle; Dipalmitoyl phosphatidyl choline; Gramicidin

1. Introduction

Lipidic bilayer constitute the matrix of biological membranes in which are inserted a large number of proteins. The structure and function of these proteins depend upon the physicochemical properties of the whole structure. It has been established that a phase change from the gel state to the liquid crystalline state modifies the properties of the lipidic annulus, and drastically changes the lateral diffusion of the lipids. This can strongly affect the function of the incorporated polypeptides [1–4]. Furthermore, it can be expected that incorporation of the polypeptide chain drastically changes the dynamical and structural properties of the bilayer, particularly in the vicinity of the macromolecule. It can be said then that the incorporation of a protein depends on, but also modifies, the properties of the bilayer. In general, the lipidic matrix is stable enough as to constitute and efficient diffusion barrier, but retains its capacity to follow changes in shape and volume, and adapt to the incorporation of large molecules. This capacity of adaptation can be significantly modified by physical (temperature, pressure) and chemical (additives, chemical composition, chemical modifications) changes [5–8].

Gramicidin is a lineal polypeptide that has been widely employed as a model molecule to mimic the behavior of intrinsic membrane proteins. It is constituted by 15 hydrophobic amino acids with L and D alternate conformations

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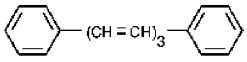
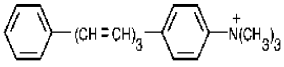
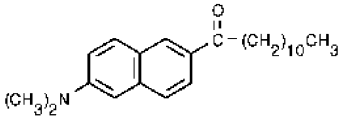
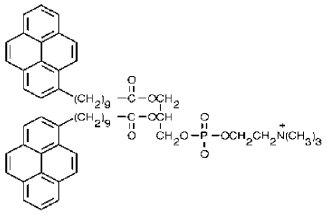
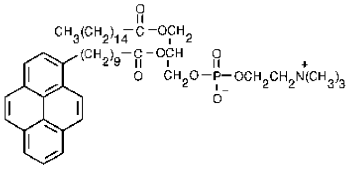
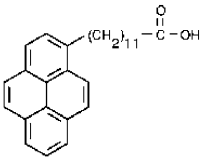
[9]. In membranes, it can form a channel constituted by two peptidic chains joint by hydrogen bonds comprising the N-terminal group [10]. The length of the dimer is ca. 26–30 Å, a distance that matches the width of the hydrophobic part of lipidic bilayers. This allows the formation of an hydrophilic pore, with the side group of the amino acids in close contact with the lipid chains [11,12]. This strong association of the polypeptide and the lipids must lead to a strong interrelation between the properties of the guest and the host. Gramicidin incorporation must affect then the structure, organization and dynamics of the lipids, and these changes should modulate the dynamics, conformation and degree of aggregation of the polypeptide. Numerous studies carried out in model membranes of phosphatydil choline (PC) support this strong interrelation. In particular, it has been established that the physical state and the packing of the bilayer conditions the incorporation, behavior and dynamic properties of the incorporated guest. For example, it has been shown that the polypeptide preferentially partitions towards the more fluid phase [13], where it presents a higher rate of axial reorientation [14,15]. Similarly, incorporation of the polypeptide to bilayers in the gel state increases the mobility of the lipids [16,17].

The effect of the polypeptide incorporation upon the bilayer properties has been evaluated in the bulk of the membrane and mostly when it is in the gel state. In the present communication we have evaluated the effect of the additive upon the properties of the bilayer sensed at different depths, both in the gel and liquid crystalline states. Also, we attempt to evaluate the average properties of the bilayer and those sensed by probes in the vicinity of the incorporated polypeptide. To achieve these aims, we have used a series of extrinsic fluorescent probes localized at different depths (6-dodecanoyl-2-dimethyl aminenaphthalene (Laurdan), 1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), 1,6-diphenyl-1,3,5-hexatriene (DPH), pyrenebutyric acid (PBA), pyrenedodecanoic acid (PDA), 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (Py–C_{10}–PC) and 1,2-bis-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (bis-Py–C_{10}–PC).

Table 1
Probes employed and bilayer properties sensed

Name	Structure	Localization	Property measured	Information
DPH		Inner part of the bilayer	Steady State anisotropy Limiting anisotropy Order parameter Rotational correlation	Microviscosity Microfluidity
TMA-DPH		Beginning of the acyl chains	Steady state anisotropy Limiting anisotropy Order parameter Rotational correlation	Microviscosity Microfluidity Mobility of the chains
Laurdan		Hydrophobic–hydrophilic interface	GP	Relaxation time of the water molecules Water penetration/ mobility
bis-Py–C_{10}–PC		Inner part of the bilayer	Intramolecular excimer Excimer–monomer Ratio	Lateral diffusion of the chains Oxygen diffusion/ concentration
Py–C_{10}–PC		Inner part of the bilayer	Intermolecular excimer–monomer ratio Quenching by oxygen Lifetime	Rotational mobility of the acyl chain Oxygen diffusion/ solubility Exposure to the solvent
PDA		Inner part of the bilayer	Intermolecular excimer–monomer ratio Lifetime Quenching by oxygen	Mobility lipid chain Oxygen diffusion/solubility Exposure to the solvent

These probes are predominantly distributed at the interface (Laurdan), at the level of the beginning of the alkyl chains (TMA-DPH, PBA), and at the inner parts of the bilayer (DPH, Py–C_{10}–PC, bis-Py–C_{10}–PC, PDA). Also, the properties of the bilayer in the vicinity of the incorporated gramicidin were tested by looking to the fluorescence emitted by the probes after its excitation by resonant energy transfer (RET) from the polypeptide tryptophan groups. A summary of the probes employed and the information by them provided is given in Table 1.

2. Material and methods

2.1. Reagents

L-α-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), gramicidin D (Dubos) from *Bacillus brevis*, and acrylamide were obtained from Sigma Chemical (St. Louis, MO, USA). Chloroform, methanol, ethanol, trifluoroethanol (TFE), and tetrahydrofurane (THF) were from Merck (Darmstadt, Germany), and dimethylformamide (DMF) from Aldrich Chemical (Milwaukee, WI, USA). The probes, DPH, TMA-DPH, Laurdan, PDA, PBA, Py–C_{10}–PC, and bis-Py–C_{10}–PC were obtained from Molecular Probes (Oregon, USA).

2.2. Experimental procedures

2.2.1. Preparation of large unilamellar vesicles

Multilamellar liposomes were prepared by vortexing with buffer a film of lipids deposited in a round-bottom flask. Large unilamellar vesicles (LUVs) were prepared by extruding the multilamellar liposomes [18,19], after five freezing and thawing cycles [20]. Extrusion was performed through two polycarbonate filters (400 nm pore) (Nucleopore, Corning Costar, Cambridge, MA, USA), employing nitrogen as pressurizing gas, at a temperature of 10 °C above the melting temperature of the bilayer. The vesicles were prepared in buffer phosphate 10 mM, 150 mM NaCl, pH 7.4, at final lipid concentrations between 0.3 and 1.5 mM.

2.2.2. Incorporation of the fluorescent probes to LUVs

DPH, TMA-DPH, PDA and PBA, dissolved in DMF, and Laurdan, dissolved in ethanol, were incorporated after the LUVs preparation. A small aliquot (less than 0.5%

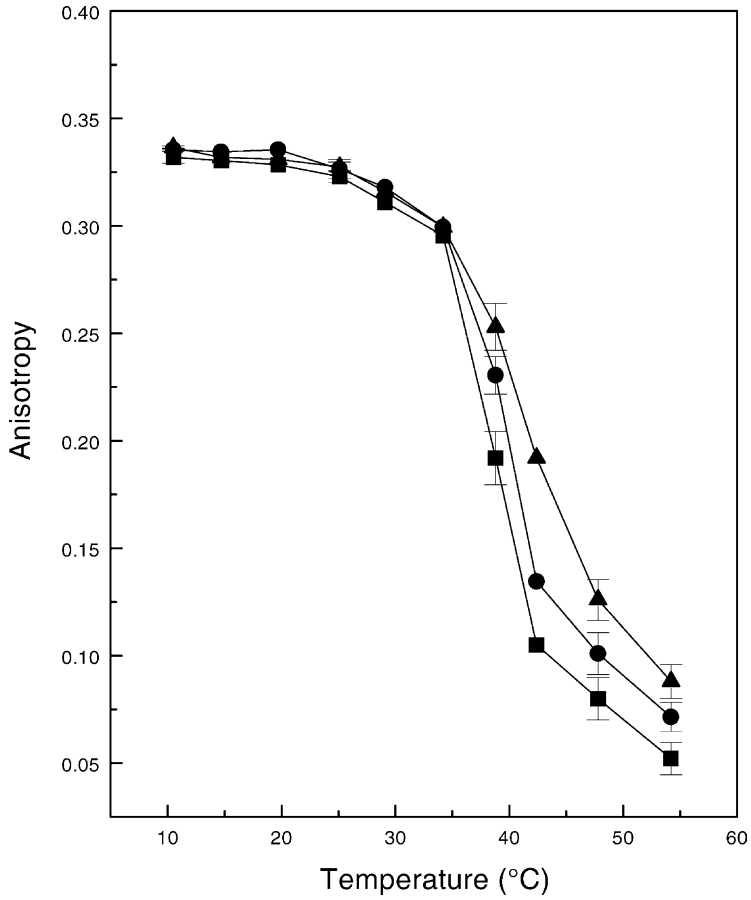


Fig. 1. Gramicidin effect upon DPH anisotropy in DPPC vesicles. Data are given as anisotropy values as a function of temperature both in absence of added gramicidin (■) and in presence of 2% (M/M) gramicidin (●) and 5% (M/M) gramicidin (▲).

in volume) of a concentrated solution was added to the LUVs suspension, and incubated by 60 min at temperatures above the bilayer transition temperature. Py–C_{10}–PC and bis-Py–C_{10}–PC [21] were deposited together with the lipids prior to preparation of the multilamellar liposomes.

The ratio probe/lipids was 1:800. Higher ratios (3:100) were employed for Py–C_{10}–PC in order to visualize the formation of intermolecular excimers. Lifetimes were measured under conditions that leading to monomeric emission only. These concentrations were 0.5 μM for PDA and PBA, and 0.001 mol% for Py–C_{10}–PC [22]. Acrylamide (0.1 M) was added to the solutions comprising PBA in order to quench all the fluorescence arising from the external solvent. This acrylamide concentration barely modifies the bilayer properties and does not quench the emission arising from bilayer incorporated probes [23].

2.2.3. Steady state fluorescence measurements

2.2.3.1. Fluorescence anisotropy measurements. Fluorescence anisotropy measurements were carried out in a phase and modulation spectrofluorimeter (GREG-200, ISS, Champaign, IL, USA) with a L type set up with Glan Thompson prism polarizers in the excitation and emission beams. Excitation was performed with a Xe arc lamp and a monochromator set at 360 nm. Two cut-off filters, Schott KV-399 and GG-420, were introduced into the path of the emission beam.

2.2.3.2. Fluorescence spectra. Fluorescence spectra were recorded in a photon counter spectrofluorimeter (Fluorolog, Spex, Metuchen, NJ, USA) employing a 300 W Xe lamp. Slits were set at 20 (excitation) and 2 nm (emission).

The ratio between the excimer and monomer emission of the pyrene derivatives, I_{exc}/I_{mon}, was equated to the ratio of the emission intensities measured at 480 and 374 nm. Direct excitation of the extrinsic chromophores was achieved by irradiation of the sample with light of 344 nm. RET experiments were performed by selective excitation of gramicidin Trp groups with light of 290 nm.

The position of Laurdan fluorescence spectra was evaluated from the fluorescence intensities measured at 490 and 440 nm [24]. The position of the spectra was characterized by the value of the generalized polarization (GP) parameter,

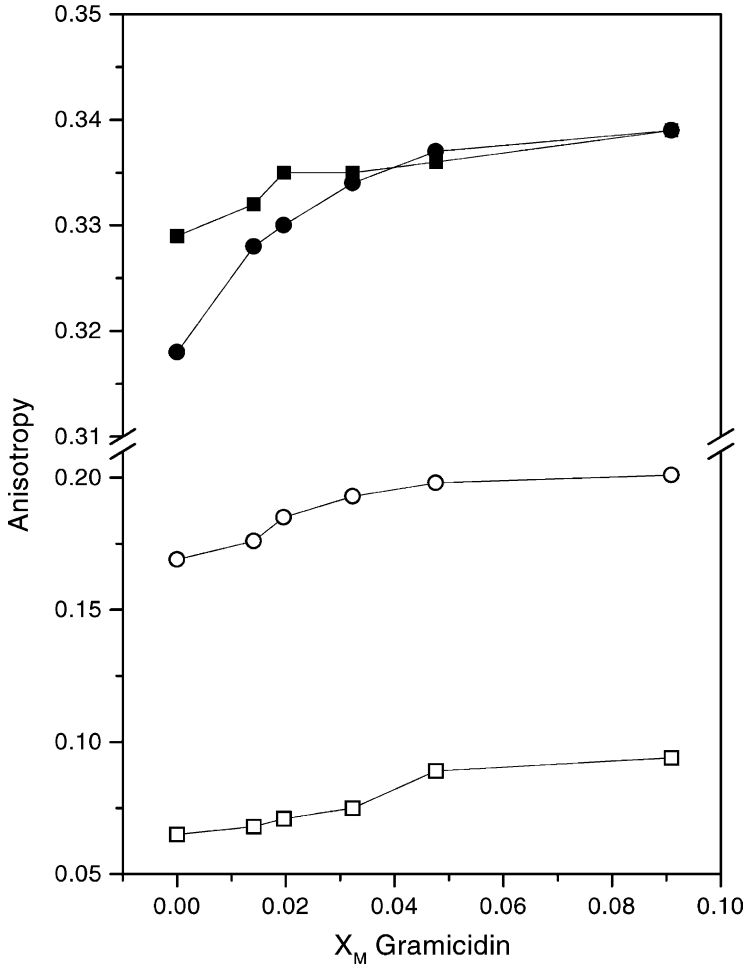


Fig. 2. Gramicidin effect upon the anisotropy of DPH and TMA-DPH incorporated to DPPC LUVs. Data are given at temperatures below (20 °C) and above (54 °C) the vesicles transition temperature. (■) DPH, 20 °C; (□) DPH, 54 °C; (●) TMA-DPH, 20 °C; (○) TMA-DPH, 54 °C.

defined by

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

The GP associated to the direct excitation of Laurdan was obtained employing light of 360 nm in the excitation beam. RET values were obtained employing light of 290 nm.

2.2.4. Time resolved fluorescence measurements

Lifetimes and time resolved anisotropy measurements were carried out in a multifrequency phase and modulation spectrofluorimeter (GREG-200, ISS Urbana Champaign, IL, USA). Excitation was performed employing the 325 nm line of a He–Cd laser (40420NB Liconix, Santa Clara, CA, USA) and a pockell cell modulated at frequencies between 0.5 and 250 MHz. Measurements were carried out at 10 frequencies in the range of 0.5–10 MHz (pyrene derivatives) or 2–110 MHz (DPH and TMA-DPH). Emitted fluorescence was filtered through a WG-360 (pyrene derivatives) or GG-420 (DPH derivatives) cut-off filter. Lifetime measurements were carried out with the emission polarizer set up at 54.7° [25–28] and employing $(Me)_2$POPOP in ethanol (lifetime 1.45 ns) as reference. Time resolved anisotropy measurements were carried out by the method described by Weber [27]. Glan Thompson prism polarizers were employed to select the vertical and horizontal light components.

Table 2
Lifetimes (τ) and width of the distribution (w) for the fluorescence of DPH and TMA-DPH measured at 20 and 54 °C

Gramicidin (%)	20 °C			54 °C		
	τ (ns)	w	χ^2	τ (ns)	w	χ^2
DPH						
0	10.4	1.29	1.25	7.9	0.71	2.12
2	10.9	1.03	1.89	7.7	1.54	3.01
5	10.7	1.34	2.02	7.2	1.09	2.41
TMA-DPH						
0	6.5	1.55	1.45	3.1	1.18	2.01
2	6.4	1.69	1.79	3.0	0.63	2.35
5	5.6	2.10	2.47	2.7	0.72	2.12

Table 3
Values of the limiting anisotropy (r_∞) and rotational correlation time (ϕ) measured for DPH and TMA-DPH at 20 and 54 °C

Gramicidin (%)	20 °C			54 °C		
	r_∞	ϕ	χ^2	r_∞	ϕ	χ^2
DPH						
0	0.31	3.83	0.81	0.03	1.67	2.34
2	0.31	–	0.49	0.04	1.57	1.39
5	0.30	9.27	0.93	0.06	1.74	0.77
TMA-DPH						
0	0.32	3.08	1.89	0.07	2.91	2.57
2	0.31	4.63	2.55	0.11	2.40	2.83
5	0.31	2.80	2.97	0.12	2.33	3.18

In all measurements, data were taken till the standard deviation of the phase and modulation measurements were, at each modulation frequency, smaller than 0.2° and 0.004°, respectively. The results were analyzed according to the Global Program (Global Unlimited, Urbana, IL, USA) [29]. For lifetimes evaluation, the results were analyzed employing a distribution model [29,30]. Independently determined lifetimes were incorporated to the evaluation of the time resolved anisotropy. The treatment provides the values of the rotational correlation time (ϕ), related

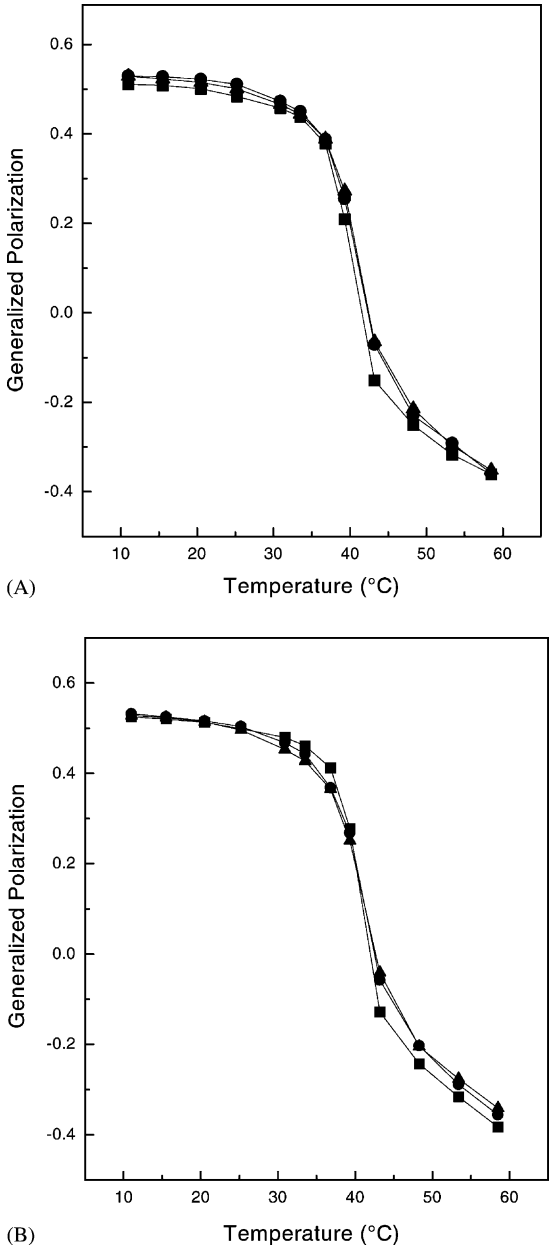


Fig. 3. Gramicidin effect upon the GP of Laurdan incorporated to DPPC LUVs. Data are given as a function of temperature following direct irradiation of Laurdan (A) or by RET from protein Trp groups (B). GP values were obtained in the absence of added gramicidin (■) and in presence of 2% (M/M) gramicidin (●) and 5% (M/M) gramicidin (▲).

to the dynamics of the probe rotation, and the limiting anisotropy (r_∞), related to the order parameter of the lipidic packing.

3. Experimental results

3.1. Fluorescence anisotropy of DPH and TMA-DPH

The data given in Fig. 1 show the effect of gramicidin addition upon the fluorescence anisotropy of DPH incorporated to DPPC LUVs measured in the 10–55 °C temperature range. In this figure are shown fluorescence anisotropy values obtained in absence of gramicidin and in presence of 2 and 5% (in a molar basis) of the additive. The results indicate that the presence of the polypeptide displaces the phase transition temperature of the bilayer towards higher temperatures. This is contrary to the behavior expected if the solute preferentially partitions in the liquid crystalline domains [13]. Another noticeable feature of the data given in Fig. 1 is the fact that the transition seems to be broader in the presence of the polypeptide, particularly at the highest gramicidin concentration considered. The change in anisotropy with the additive concentration, measured below (20 °C) and above (54 °C) the bilayer phase transition is shown in Fig. 2. In this figure are also included the results obtained employing TMA-DPH, a probe localized at (or near) the bilayer interface. The data of this figure show that the anisotropy of both the probes slightly increases when the fraction of gramicidin increases. When the bilayer is in the gel state, the probe anisotropy is close to the limiting anisotropy (r_0 = 0.38) and, at low gramicidin content, is slightly smaller for TMA-DPH than for DPH. On the other hand, at temperatures above the bilayer phase transition, the anisotropy of TMA-DPH is considerably larger than that of DPH, a result that can be explained in terms of the anchor effect of the charged head group in TMA-DPH.

In order to assess which factors condition the values of the anisotropy and the origin of the effect of the polypeptide, we measure the lifetime of the probe and the width of the Lorentzian distribution. The data obtained are given in Table 2. These data show that raising the temperature from 20 to 54 °C reduces the lifetime of the probe. This reduction, that can be associated to an increased effect of

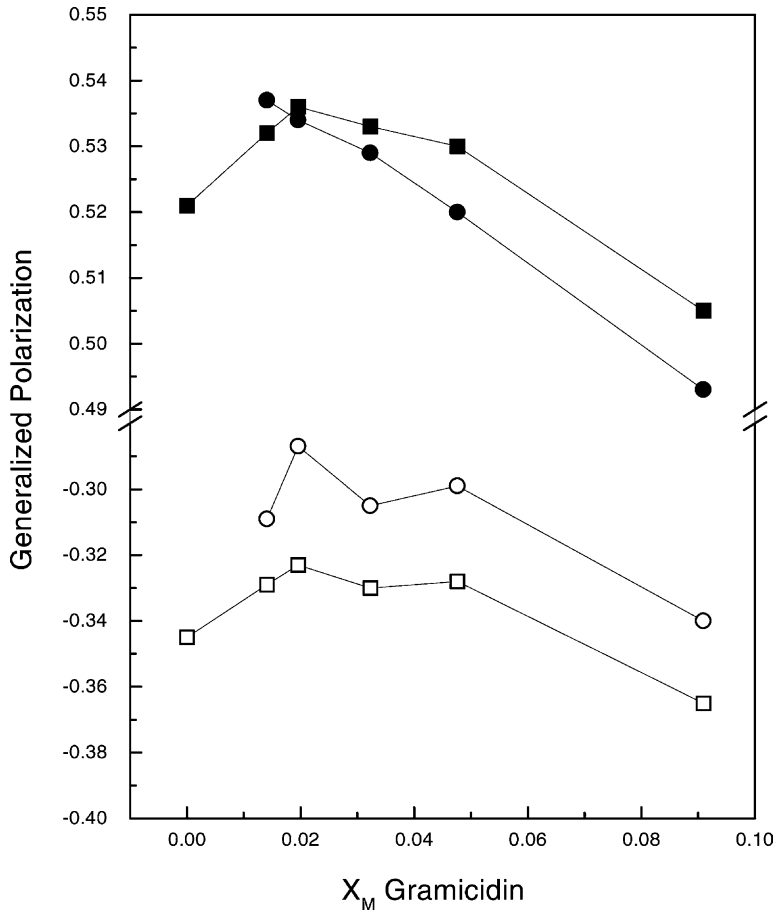


Fig. 4. Gramicidin effect upon the GP of Laurdan incorporated to DPPC LUVs. Data are given as a function of gramicidin concentration at temperatures below (20 °C) and above (54 °C) the vesicles transition temperature by direct and RET excitation. (■) Direct irradiation, 20 °C; (□) direct irradiation, 54 °C; (●) RET irradiation, 20 °C; (○) RET irradiation, 54 °C.

water molecules [31], seems to be larger for TMA-DPH than for DPH, as expected from their relative exposition to the solvent. This factor can also explain the considerably shorter lifetime of TMA-DPH over all the conditions considered. With regard to the effect of gramicidin addition, it elicits only a small decrease in the probes lifetimes, particularly for TMA-DPH, where the effect was observed for both above and below the bilayer phase transition temperature. This could be explained either by a displacement of the probe to more polar region and/or by a partial quenching by the Trp groups of the polypeptide. With regard, to the width of the lifetime distribution, they are relatively small and do not show a clear trend with the polypeptide concentration. This shows that the microenvironment of the probes is rather homogeneous, even in presence of 5% gramicidin. However, it is interesting to note that gramicidin addition seems to have an opposite effect on the width of the distribution for TMA-DPH below and above the phase transition temperature.

Time resolved anisotropy measurements allow an independent evaluation of the bilayer order parameter and the probe rotational time. The values obtained are collected in Table 3. The data of this table show that at 20 °C the order parameter remains high, irrespective of gramicidin addition. This applies both to the region near the interface and the inner parts of the bilayer. Raising the temperature produces a noticeable reduction in r_{∞} and, hence, in the order parameter. This reduction is considerably larger for DPH, a result that could explain the low values of the anisotropies measured for this compound under steady state irradiation. For both probes, the presence of the polypeptide reduces the width of the probe movement, as evidenced by an increase in the limiting anisotropy. On the other hand, the effect of gramicidin upon the rotational correlation time is less clear, being irrelevant for DPH and showing a small but significant decrease for TMA-DPH. For this probe, the presence of the polypeptide leads then to a faster movement of the probe in a more restricted space.

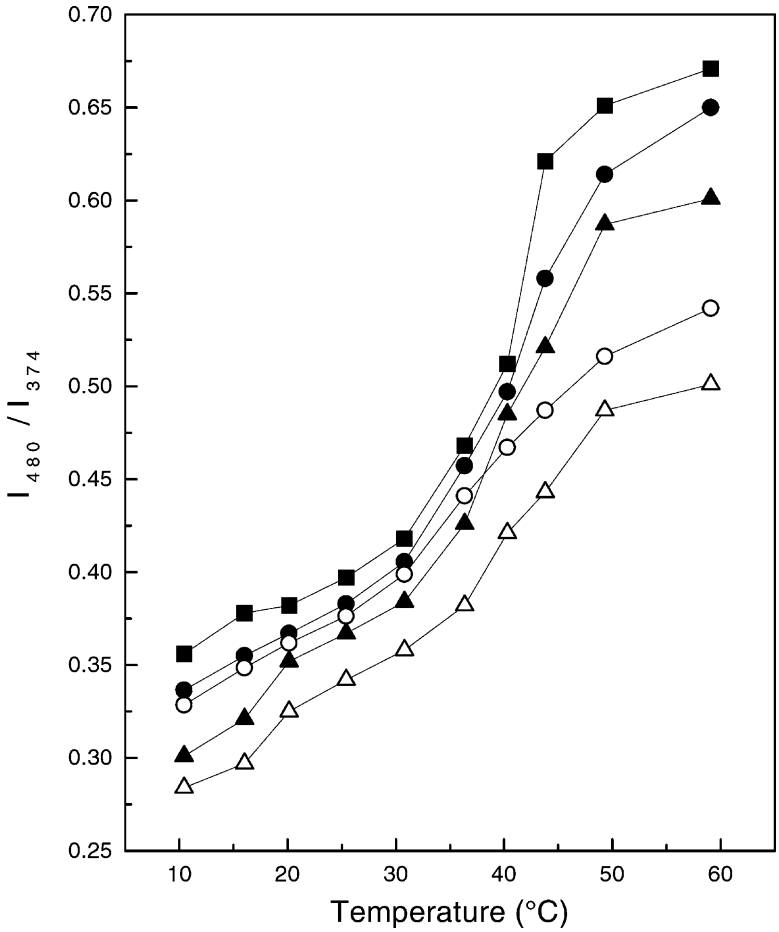


Fig. 5. Gramicidin effect upon the excimer to monomer ratio of Py–C_{10}–PC incorporated to DPPC LUVs. Data are given as a function of temperature following direct irradiation of the pyrenyl group (excitation at 344 nm) or by RET from protein Trp groups (excitation at 290 nm). Fluorescence intensities ratio, measured at 480 and 374 nm, were obtained in the absence of added gramicidin and in presence of 2% (M/M) gramicidin and 5% (M/M) gramicidin. (■) Without gramicidin, direct irradiation; (●) 2% gramicidin, direct irradiation; (○) 2% gramicidin, RET irradiation; (▲) 5% gramicidin, direct irradiation; (△) 5% gramicidin, RET irradiation.

3.2. Laurdan GP

Laurdan GP changes with temperature (Fig. 3) in a fashion similar to that of DPH anisotropy. However, a noticeable difference with DPH data is a barely noticeable displacement of the phase transition temperature with gramicidin addition. The data of this figure show that the GP values obtained, both by direct irradiation or in RET experiments, closely correlate with the fluidity of the bilayer. The effect of gramicidin addition, at temperatures below and above the bilayer transition temperature, is shown in Fig. 4. These data show that GP values, when Laurdan is randomly excited by direct irradiation, first increase and afterwards decrease when the polypeptide concentration increases. This behavior is observed both at 20 and 50 °C, and indicates that low (ca. 2%) gramicidin decreases the polarity of the probe microenvironment and/or increases the relaxation time of the water molecules. The effect is reversed at higher polypeptide concentrations. Furthermore, a comparison of the results obtained by direct or RET excitation show that, particularly at high polypeptide concentrations, the water penetration/mobility is higher in the protein annulus at low temperatures, while the opposite takes place above the bilayer phase transition temperature.

3.3. Intermolecular excimer formation

Pyrene derivatives, such as Py–C_{10}–PC, can form intermolecular excimers in a diffusion controlled process. The rate of the excimer formation, and hence the ratio between the excimer and monomer emission, depends upon the lateral diffusion of the probes and is a measure of the microviscosity of the media. Values of the ratio between the intensities measured at 480 and 374 nm, that can be considered as a measure of the excimer to monomer ratio, are given in Fig. 5 as a function of the temperature. The results given in this figure correspond to those obtained in the control LUVs and those obtained in the presence of 2 and 5% gramicidin, both by direct and RET excitation. When excited by the RET mechanism employing light of 290 nm, direct excitation of the pyrene groups leads to less than 10% of the total emission. This value was obtained by irradiating

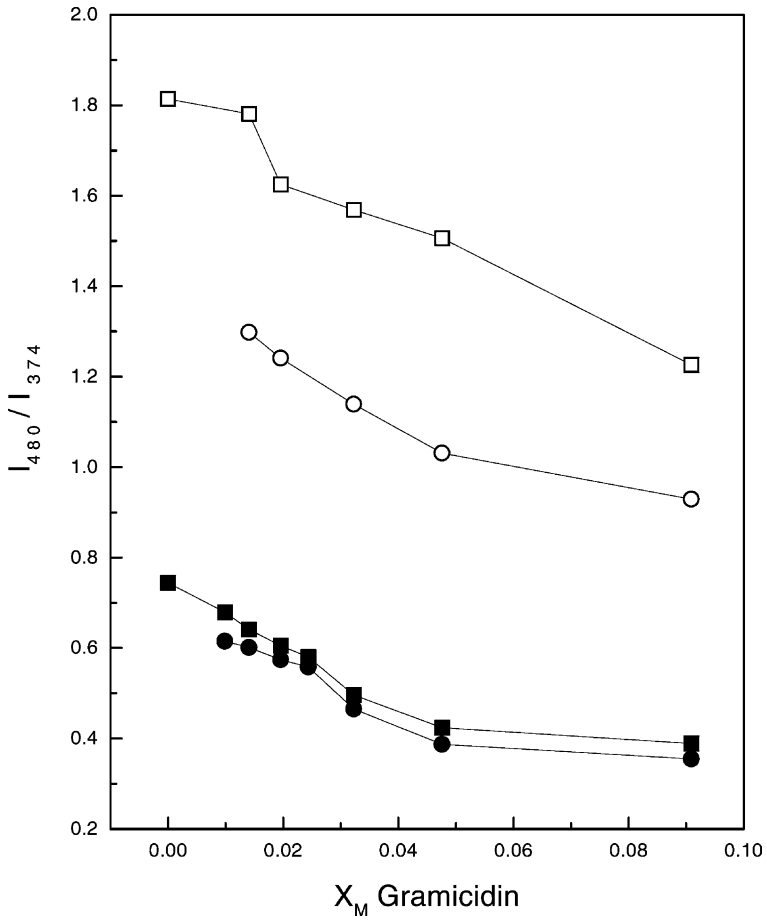


Fig. 6. Gramicidin effect upon the excimer to monomer ratio of Py–C_{10}–PC incorporated to DPPC LUVs. Data are given as a function of gramicidin concentration following direct irradiation of the pyrenyl group (excitation at 344 nm) or by RET from protein Trp groups (excitation at 290 nm). Fluorescence intensities ratio, measured at 480 and 374 nm, were obtained at temperatures below (20 °C) and above (54 °C) the vesicles transition temperature. (■) Direct irradiation, 20 °C; (□) direct irradiation, 54 °C; (●) RET irradiation, 20 °C; (○) RET irradiation, 54 °C.

the samples, in absence of polypeptide, with light of 290 nm and comparing the intensity of the fluorescence measured at 370 nm with that obtained when the irradiation is carried out in the presence of the polypeptide (data not shown).

The data given in Fig. 5 present interesting features. In the first place, the profile obtained in the control vesicles is similar to that shown in Figs. 1 and 3, indicating that the lateral mobility of the probe follows the bilayer viscosity. However, it is remarkable that the excimer to monomer ratio strongly depends on temperature even below the phase transition. Furthermore, the change associated to the phase transition is less sharp than that measured employing other probes. As with the other probes, the presence of gramicidin displaces the phase transition towards higher temperatures and somehow broadens the temperature range of the melting process.

Another peculiar feature of the data given in Fig. 5 is that, in RET excitation, the phase transition is barely noticeable. This would indicate that the melting process does not particularly affect the lateral diffusion of the lipids in the vicinity of the polypeptide chain. Furthermore, it is important to note that, over all the temperature range, RET values are smaller than those obtained in the bulk excitation of the probe, implying a more rigid environment and/or a decreased probe concentration in the polypeptide annulus. The data given in Fig. 6 show that, over all the gramicidin concentration range considered in the present study, the presence of the polypeptide decreases the lateral diffusion of the probe, both below and above the phase transition. Similarly, it shows that, particularly in the liquid crystalline state, less excimer emission arises from the vicinity of the polypeptide. It is interesting to note that quenching of the excited monomer by the polypeptide should not affect excimer to monomer ratio. On the other hand, quenching of the excimer by the polypeptide could contribute to the decreased excimer emission. Unfortunately, the low intensity of the signal precluded an evaluation of the excimer lifetime in RET excitation experiments.

3.4. Intramolecular excimer formation

The ratio between the fluorescence intensities of bis-Py–C_{10}–PC, measured at 480 and 374 nm, reflects the

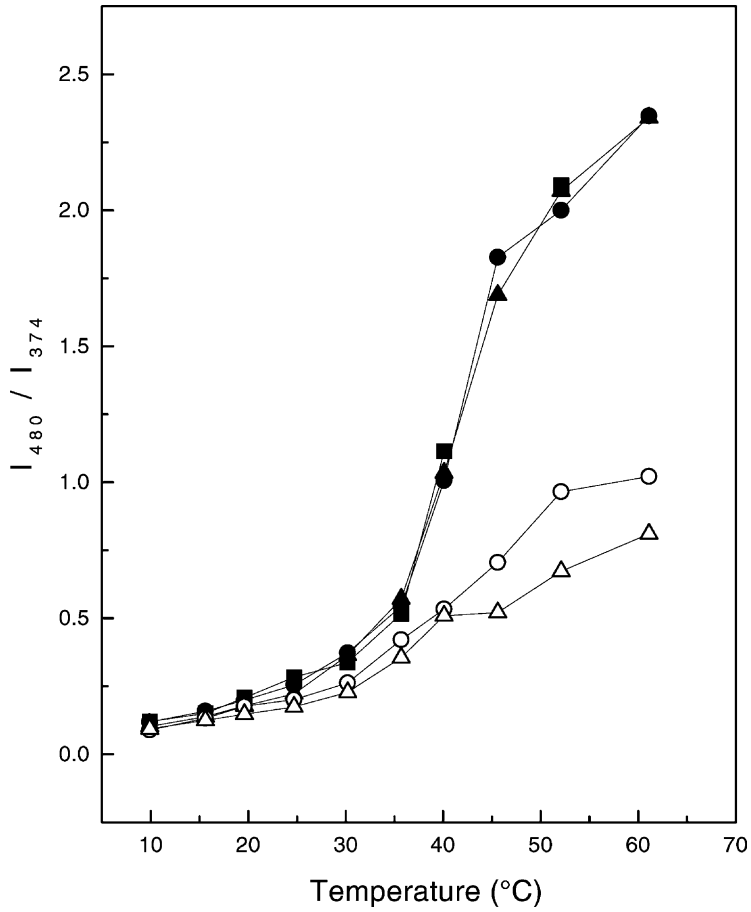


Fig. 7. Gramicidin effect upon the excimer to monomer ratio of bis-Py–C_{10}–PC incorporated to DPPC LUVs. Data are given as a function of temperature following direct irradiation of the pyrenyl group (excitation at 344 nm) or by RET from protein Trp groups (excitation at 290 nm). Fluorescence intensities ratio, measured at 480 and 374 nm, were obtained in the absence of added gramicidin and in presence of 2% (M/M) gramicidin and 5% (M/M) gramicidin. (■) Without gramicidin, direct irradiation; (●) 2% gramicidin, direct irradiation; (○) 2% gramicidin, RET irradiation; (▲) 5% gramicidin, direct irradiation; (△) 5% gramicidin, RET irradiation.

rotational mobility of the pyrenyl groups located at the end of the alkyl chain and, hence, the microviscosity of the inner parts of the bilayer. Data obtained as a function of the temperature (Fig. 7) show similar features than those obtained for the intermolecular excimer formation (Fig. 5), although the change elicited by the phase transition temperature is sharper for the intramolecular excimer. This is particularly so in presence of gramicidin. On the other hand, the results obtained by RET excitation do not clearly show the presence of the phase transition temperature. This would indicate that, in the inner parts of the membrane and in the vicinity of the polypeptide, the effect of the bilayer melting upon the rotational mobility of the pyrenyl groups is minimal.

Data obtained over all the gramicidin concentration range are shown in Fig. 8. These data show that the local properties sensed by the probe are almost independent of the polypeptide concentration, particularly in the liquid crystalline state. However, they also show that in the annulus of the polypeptide the motility is significantly reduced. This would indicate that the presence of the polypeptide is not sensed by all the bilayer, being the changes more significant in the vicinity of the additive. This conclusion applies particularly to local movements, such as those required to allow an efficient intramolecular excimer formation.

3.5. Pyrenyl group lifetime

The pyrenyl group fluorescence lifetime in the absence of excimer formation is determined by the extent of water penetration till the average solubilization site of the excited probe [32,33]. In the present work we have measured the lifetimes in the presence of different amounts of gramicidin. The measurements carried out employing PBA were carried out in the presence of 0.1 M acrylamide in order to quench all the fluorescence arising from probe molecules remaining in the dispersing solvent. The average lifetime of PBA in presence of LUVs increases from 138 to 195 ns upon addition of 40 mM acrylamide. This value remains constant when the quencher concentration increases up to 140 mM.

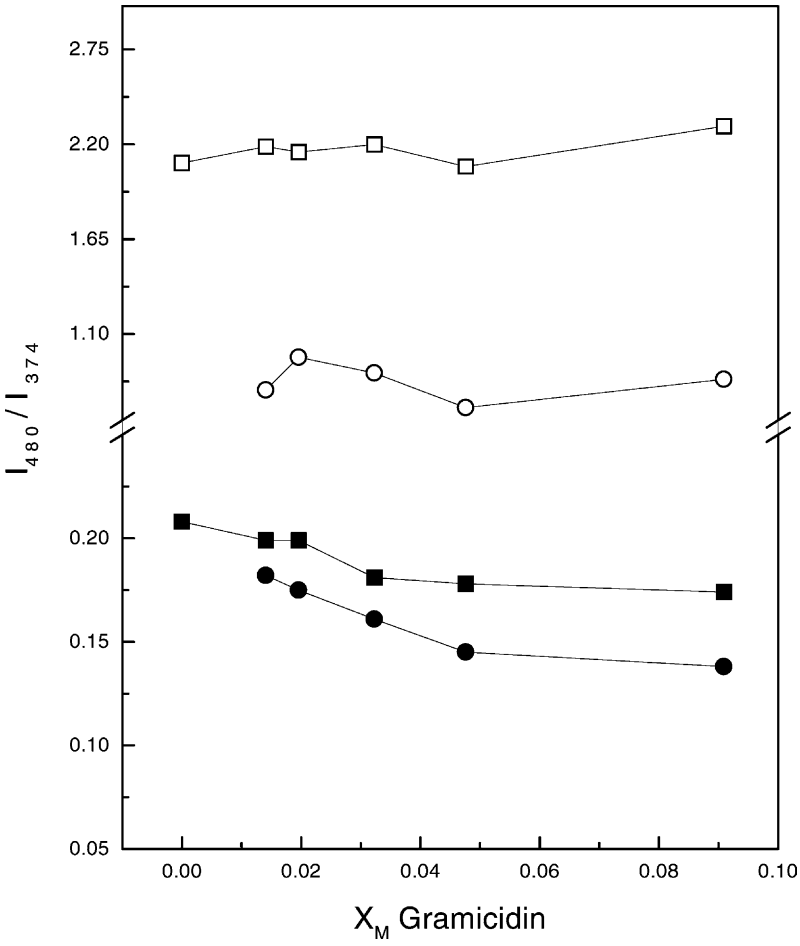


Fig. 8. Gramicidin effect upon the excimer to monomer ratio of bis-Py–C_{10}–PC incorporated to DPPC LUVs. Data are given as a function of gramicidin concentration following direct irradiation of the pyrenyl group (excitation at 344 nm) or by RET from protein Trp groups (excitation at 290 nm). Fluorescence intensities ratio, measured at 480 and 374 nm, were obtained at temperatures below (20 °C) and above (54 °C) the vesicles transition temperature. (■) Direct irradiation, 20 °C; (□) direct irradiation, 54 °C; (●) RET irradiation, 20 °C; (○) RET irradiation, 54 °C.

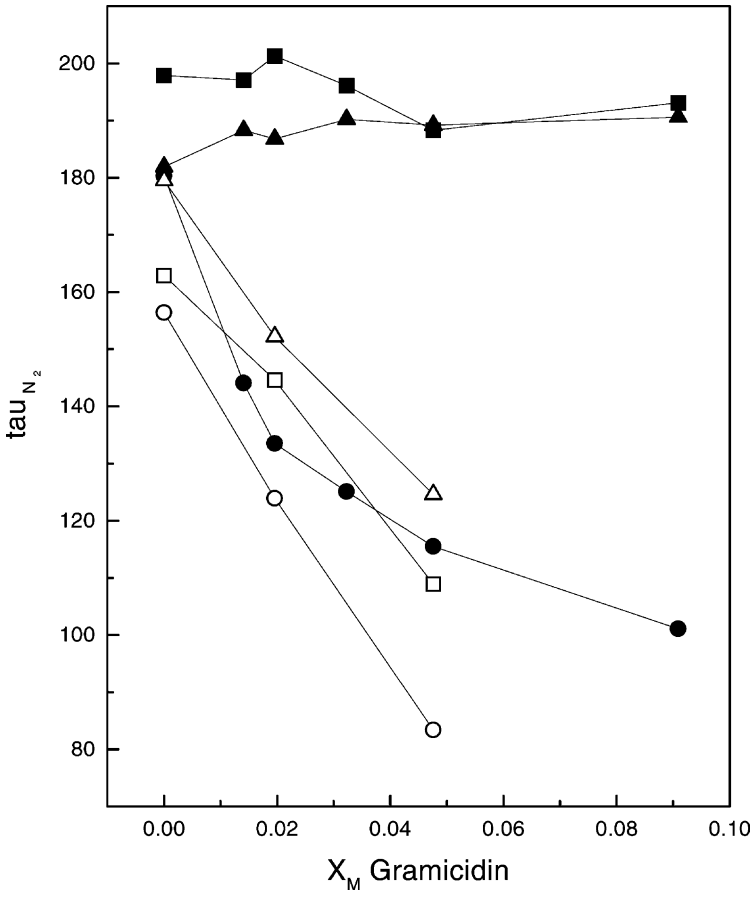


Fig. 9. Gramicidin effect upon the fluorescence lifetime of different pyrene derivatives incorporated to DPPC LUVs. Data are given as a function of gramicidin concentration following direct irradiation of the pyrenyl group (excitation at 344 nm). Results were obtained at temperatures below (20 °C) and above (54 °C) the vesicle transition temperature. (■) PDA, 20 °C; (□) PDA, 54 °C: (●) PBA, 20 °C; (○) PBA, 54 °C; (▲) Py–C_{10}–PC, 20 °C; (△) Py–C_{10}–PC, 54 °C.

This indicates that all the fluorescence arising from the aqueous phase has been quenched, and that acrylamide addition does not appreciably modify the fluorescence lifetime of the vesicle incorporated probes.

Fluorescence lifetimes measured in the presence of increasing gramicidin concentrations are shown in Fig. 9. The data obtained in the absence of gramicidin show a small tendency to shorter lifetimes at higher temperatures and when the probe is localized nearer to the interface. This is compatible with a partial quenching of the excited pyrenyl groups by water molecules [33]. At low temperatures, the effect of gramicidin addition depends on the probe. While the effect is minimal for probes deeply incorporated into the bilayer, there is a noticeable decrease in the probe fluorescence lifetime with the polypeptide concentration for PBA, whose pyrenyl group is located at intermediate positions. This could indicate the presence of bilayer defects produced by the polypeptide and/or quenching by the Trp groups, that are present at the C-terminal part of the polypeptide and hence should be present near both interfaces of the bilayer [9,12,34]. On the other hand, the effect of gramicidin upon the lifetime of the three pyrene derivatives is similar when the bilayer is in the liquid crystalline state (Fig. 9). In fact, a significant decrease in lifetime is observed, proportional to the polypeptide concentration, irrespective of the pyrenyl group localization. This can be due to a displacement of the probes towards the interface where it can be quenched by water molecules and/or Trp groups, and/or due to a deeper and faster penetration of water molecules into the more fluid bilayer [35].

3.6. Quenching of pyrene fluorescence by oxygen

The pseudo first order quenching rate constant by oxygen was obtained from the probe fluorescence lifetime in nitrogen purged and oxygen saturated solutions. The values of k_{ox} obtained by this procedure can be considered as a measure of the concentration/mobility of the oxygen molecules in the bilayer, averaged over all the pathway of the oxygen molecule during the lifetime of the excited probe [32]. This value depends then on the properties of the bilayer beyond the immediate surroundings of the excited probe.

The values of k_{ox} measured in the present work are shown in Fig. 10. These data show, as expected, a noticeable

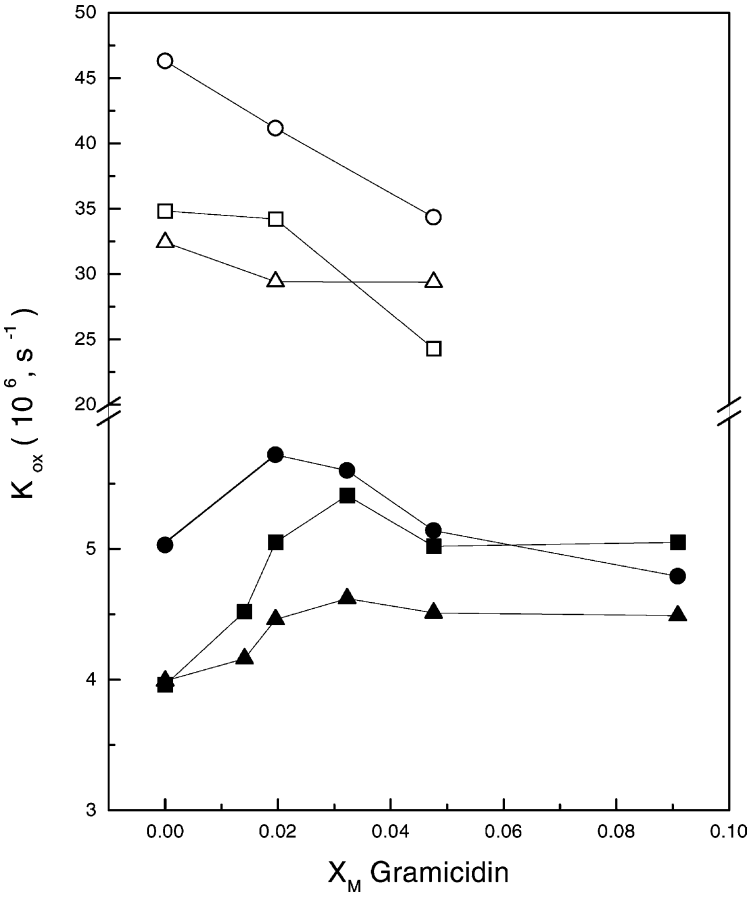


Fig. 10. Gramicidin effect upon the fluorescence quenching rate constant by oxygen of different pyrene derivatives incorporated to DPPC LUVs. Data are given as a function of gramicidin concentration following direct irradiation of the pyrenyl group (excitation at 337 nm). Results were obtained at temperatures below (20 °C) and above (54 °C) the vesicle transition temperature. (■) PDA, 20 °C; (□) PDA, 54 °C: (●) PBA, 20 °C; (○) PBA, 54 °C; (▲) Py–C_{10}–PC, 20 °C; (△) Py–C_{10}–PC, 54 °C.

increase in k_{ox} associated to the bilayer melting. Also it shows that, both below and above the transition temperature of the bilayer, k_{ox} values seem to be larger for PBA than for the probes whose pyrenyl groups are expected to be located at the interior of the bilayer. This result is contrary to that obtained employing red blood cell membranes, and could indicate that in the liposomes prevail the transversal movement of the oxygen molecules. This conclusion does not hold in the presence of large amounts of gramicidin, in particular when the bilayer is in the gel state.

Other noticeable aspects of the data given in Fig. 10 are:

i) The decrease observed in k_{ox} at high temperatures when the gramicidin concentration increases. This implies that gramicidin reduces the mobility/solubility of oxygen inside the bilayer when this is in the liquid crystalline phase.
ii) The increase observed in k_{ox} at low gramicidin concentrations when the bilayer is in the gel state. In this rigid and ordered system, the presence of low gramicidin concentrations increases the mobility/solubility of oxygen molecules.

4. Discussion

The results obtained in the present work show that gramicidin incorporation produces, on the gel phase, a small increase in the order degree of the phospholipid alkyl chains at the surface of the membrane, and over all of the membrane bilayer when it is in the crystalline phase. These results agree with reported data obtained on DMPC vesicles using ^{2}H-RMN measurements [36]. These results show that, in the crystalline phase, there is a direct correlation between the order degree of the acyl chains and gramicidin concentration. Furthermore, the effect of gramicidin on the acyl chains order degree depends on the alkyl chain length, as shown by measurements using DMPC, DPPC and DSPC [36]. The greater increase in the order of the acyl chains was observed for DMPC, while a smaller increase was found for DPPC and none in DSPC vesicles. These effects can be related to the phase transition temperature displacement to higher temperatures observed in fluorescence anisotropy measurements employing DPH and TMA-DPH as probes. The present data agree with the reported widening of the phase transition of DMPC vesicles [37]. Furthermore, the fact that the most

significant changes are observed near the interface agrees with the data obtained in the liquid crystalline phase of DMPC vesicles using time resolved fluorescence measurements with TMA-DPH and DPH-PC as fluorescent probes [3,4]. Chapman et al. [15–17] have reported, using IR and Raman spectroscopy, that gramicidin incorporation produces an increase of the lipid mobility in DMPC and DPPC vesicles in the gel phase, concluding that the disorder of the lipidic matrix increases with gramicidin concentration. Our results obtained with TMA-DPH and Py–C_{10}–PC show a tendency to increase the order degree packing and decrease the scope of short lateral diffusion displacements, both in the gel state and in the liquid crystalline phase.

Several studies have shown that gramicidin locates preferentially in fluid domains [13,39], and that there are non-specific lipid mediated interactions that depend on the lipid composition of the membrane. These lipid–polypeptide interactions produce stable conformations (association of two monomers) in the membrane in which their hydrophobic surfaces interact with the lipidic core of the membrane, and their hydrophilic surface is in contact with the polar solvent. The present results would indicate that the lipid–protein interface has different properties than the bulk of the bilayer, lending further support to the lipidic annulus hypothesis sustained by several experimental techniques [38–40]. Dynamic studies of gramicidin A interaction with DMPC vesicles show that the polypeptide reduces the fast movement of the acyl chains, but not appreciably affect the slow movements that predominates near the interface [41]. These results were ascribed to a strong interaction between acyl chain heads and the Trp residues of the polypeptide chain. This reduces the lateral diffusion of the lipidic chains, leading to a decrease in intermolecular excimer formation over all the temperature range (Fig. 6). This restriction should be particularly significant in the lipidic annulus when the bilayer is in the liquid crystalline phase. A comparison of the data obtained for the intermolecular (Fig. 6) and intramolecular (Fig. 8) excimer formation show similarities but also significant differences. In both the systems, the decrease in excimer formation associated to RET excitation is considerably larger at high temperatures, where excimer formation could involve a relative displacement of the probes over longer distances. These results agree with data obtained in a solid state ^{13}C NMR study that indicate a decrease in lateral diffusion associated to gramicidin A incorporation [42]. On the other hand, they somehow differ from the results reported by Engelke et al. [43] on the protein-dependent reduction of the pyrene excimer formation in small liposomes of EPC. In this study, similar excimer to monomer ratios were obtained by direct (336 nm) and RET (292 nm) excitation.

A noticeable difference between the data given in Figs. 6 and 8 is the different dependence with the polypeptide concentration. In fact, these data show that, while the intermolecular excimer formation is gramicidin concentration dependent, the intramolecular process is almost independent of it, particularly at high temperatures. This difference can be also related to the distinct type of diffusion required to reach the excimer conformation. In particular, lateral diffusion must involve longer distance displacements, a process particularly sensitive to the polypeptide intramembrane concentration.

The above discussion show that the effects produced by gramicidin incorporation into lipidic bilayers are complex and difficult to describe at the molecular level. The present data emphasizes the point that the effect produced depends upon the property being sensed, the position of the sensor (regarding the proximity of the interface and/or the incorporated polypeptide chain), the phase of the bilayer, and the gramicidin/lipid ratio. It is very difficult then to make general statements regarding the effect of the polypeptide upon the properties of the bilayer. Most of the properties measured in the present work somehow sense the "microviscosity" of the bilayer (DPH and TMA-DPH anisotropy, excimer to monomer ratio of pyrene derivatives, quenching by oxygen) or the extent of water molecules penetration (pyrene lifetimes) and relaxation times (GP of Laurdan). However, it has to be considered that even properties related to "microviscosity" can sense different types of motion: lateral diffusion in Py–C_{10}–PC and PDA, relative movement of the acyl chains in bis-Py–C_{10}–PC, rotation in DPH and TMA-DPH, and diffusion and solubility of the oxygen molecule in pyrene derivatives fluorescence quenching. This has to be taken into account when discussing data obtained while employing different probes that apparently sense the same property of their environments. In spite of these difficulties, we shall try to summarize the results obtained under the following conditions (Table 4):

i) near the interface at low temperature (gel phase);
ii) near the interface at high temperature (liquid crystalline phase);
iii) inner parts of the membranes at low temperature;
iv) inner parts of the membranes at high temperatures.

Items (i) and (ii) correspond to the data obtained employing DPH, Laurdan and PBA, while items (iii) and (iv) correspond to the data obtained with the other fluorescent probes.

The most consistent changes associated to gramicidin incorporation are those observed at the inner parts of the membrane in the liquid crystalline state. Under these conditions, most of the probes report a decrease in the order and fluidity of the bilayer. In fact, a decrease in the limiting anisotropy of DPH, extent of excimer formation (particularly for the intramolecular excimer formation) and quenching rate constant by oxygen is observed. Furthermore, with RET excitation there is a decrease in excimer formation, indicating a more restricted mobility of the acyl chains in the vicinity of the polypeptide. However, it is remarkable that there are no significant changes in DPH rotational correlation times.

The changes observed in the inner parts of the bilayer in the gel state and interfacial regions (both below and above the phase transition temperature) are more complex

Table 4
Summary of results

Position	Phase	Probe	Changes observed with increasing gramicidin percentage
Near the interface	LC	DPH-TMA	Increase in r_∞, decrease in Φ
		Laurdan	Increase and decrease in GP < GP in RET
		PBA	Decrease in τ_{N2}, decrease in k_{ox}
	Gel	DPH	Increase in r
		Laurdan	Increase and decrease in GP < GP in RET
		PBA	Decrease in τ_{N2}, increase and decrease in k_{ox}
Inner parts	LC	DPH	Increase in r, increase in r_∞
		bis-Py–C_{10}–PC	No change < in RET
		Py–C_{10}–PC	Decreased excimer < in RET, decrease in τ_{N2}, decrease in k_{ox}
		PDA	Decrease in τ_{N2}, decrease in k_{ox}
	Gel	DPH	Increase in r, increase in Φ
		bis-Py–C_{10}–PC	Decreased excimer < in RET
		Py–C_{10}–PC	Decreased excimer equal in RET, no change in τ_{N2}, increase in k_{ox} (afterwards constant)
		PDA	Small increase in τ_{N2}, increase and decrease in k_{ox}

and probe dependent. In particular, in the central parts of the bilayer in the gel phase the limiting anisotropy remains very high and nearly constant; there is an increase in DPH rotational correlation time, and a decrease in intermolecular excimer formation. These changes would suggest a decrease in the mobility of the probes associated to gramicidin incorporation. However, the intramolecular excimer formation is almost independent of the gramicidin addition, and the quenching for oxygen first increases and afterwards tends to decrease when the gramicidin concentration increases. It is interesting to note that, in this regard, the effect of gramicidin incorporation is very similar to that reported for a branched alkanol (2,6-dimethyl-4-heptanol). For this solute, small amounts of alkanol increase k_{ox}, while larger amounts decrease its value significantly [35]. The differences observed when the probes are directly or RET excited are also probe dependent, since no significantly differences are observed for the intermolecular excimer, while a decreased intramolecular excimer formation is observed that employed RET excitation.

The response of the interface properties to the polypeptide incorporation is more sensitive to the probe characteristics and to the probe being employed. This can be related to the intrinsic inhomogeneity of the region and/or to different localizations of the extrinsic sensors. Below the phase transition, gramicidin incorporation barely changes the order parameter (TMA-DPH) and first rigidizes (at 2%) and afterwards fluidizes (5%) the interface (Table 1). Similar behavior has been reported for alkanols in DPPC vesicles [35]. On the other hand, gramicidin incorporation increases the value of k_{ox} only in the low concentration range. The effect of the additive upon the water relaxation rate is also gramicidin dependent. The data of Fig. 4 show a moderate decrease in water relaxation at low gramicidin, followed by a decrease at higher gramicidin/lipid ratios. In this sense, the response of Laurdan to gramicidin incorporation closely resembles that of the rotational correlation time of TMA-DPH (Table 2). A remarkable feature of the data obtained near the interface and at low temperature are the slightly lower values of Laurdan GP in the RET excitation, a result that would suggest a slightly higher water relaxation rate in the vicinity of the polypeptide chain.

The changes at the interface level at high temperatures would then indicate an increase in the order parameter with very little changes in the rotational correlation time (Table 3), a monotonic decrease in the oxygen quenching constant (Fig. 10), a monotonic decrease in pyrene derivatives lifetime, and a small increase in Laurdan GP at intermediate gramicidin concentrations (Fig. 4). GP values obtained by RET excitation are higher than those obtained by direct irradiation, over all the gramicidin range. Most of these data indicate a moderate increase in rigidity and order, particularly in the vicinity of the polypeptide chain.

Acknowledgements

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References

[1] A. Dannenberg, J. Kavecansky, S. Scarlata, D. Zakim, Biochemistry 29 (1990) 5961.
[2] S. Scarlata, Biochemistry 30 (1991) 9853.
[3] J. Müller, G. van Ginkel, E. van Faasen, Biochemistry 34 (1995) 3092.
[4] J. Müller, G. van Ginkel, E. van Faasen, Biochemistry 35 (1996) 488.
[5] P. Yeagle, FASEB J. 3 (1989) 1833.
[6] B. Lewist, D. Engelman, J. Mol. Biol. 166 (1983) 211.
[7] P. Sotomayor, M. Bagnara, M. Soto, E. Abuin, E.A. Lissi, Bol. Soc. Chil. Quím. 38 (1993) 221.
[8] P. Sotomayor, F. Aguilar, A. Campos, E.A. Lissi, Bol. Soc. Chil. Quím. 41 (1996) 93.

[9] K. Rinehart Jr., J. Cook Jr., H. Meng, K. Olson, R. Pandey, Nature 269 (1977) 832.
[10] O. Andersen, Annu. Rev. Physiol. 46 (1984) 531.
[11] D. Urry, Proc. Natl. Acad. Sci. USA 68 (1971) 672.
[12] D. Urry, M. Goodall, J. Glickson, D. Mayers, Proc. Natl. Acad. Sci. USA 68 (1971) 1907.
[13] S. Wang, E. Martin, J. Cimino, G. Omann, M. Glaser, Biochemistry 27 (1988) 2033.
[14] B. Cornell, F. Saparovic, A. Baldassi, R. Smith, Biophys. J. 53 (1988) 67.
[15] D. Chapman, B. Cornell, A. Eliasz, A. Perry, J. Mol. Biol. 113 (1977) 517.
[16] E. Weidekamm, E. Bamberg, D. Brdiczka, G. Wildermuth, F. Macco, R. Weber, Biochim. Biophys. Acta 464 (1977) 442.
[17] H. Susi, J. Sampugna, J. Hampson, J. Ard, Biochemistry 18 (1979) 297.
[18] D. Mayer, M. Hope, P. Cullis, A Janoff, Biochim. Biophys. Acta 817 (1985) 193.
[19] D. Mayer, M. Hope, P. Cullis, Biochim. Biophys. Acta 858 (1986) 161.
[20] M. Hope, M. Bally, G. Webb, Biochim. Biophys. Acta 812 (1988) 55.
[21] M. Vauhkonen, M. Sassaroli, P. Somerharju, J. Eisinger, Biophys. J. 57 (1990) 291.
[22] M. Sassaroli, M. Vauhkonen, D. Perry, J. Eisinger, Biophys. J. 57 (1990) 281.
[23] P. Sepúlveda, G. Gallardo, E.A. Lissi, J. Colloids Interf. Sci. 152 (1992) 104.
[24] T. Parassasi, E. Gratton, J. Fluoresc. 5 (1995) 59.
[25] R. Spencer, G. Weber, Ann. NY Acad. Sci. 158 (1969) 361.
[26] R. Spencer, G. Weber, J. Chem. Phys. 52 (1970) 1654.
[27] G. Weber, J. Chem. Phys. 66 (1977) 4081.
[28] J. Lakowicz, Principles in Topics in Fluorescence Spectroscopy, Vol. II, Plenum Press, New York, 1984, pp. 241–305.
[29] D. Jameson, E. Gratton, R. Hall, Appl. Spectrosc. Rev. 20 (1984) 55.
[30] R. Fiorini, M. Valentino, S. Wang, M. Glaser, E. Gratton, Biochemistry 26 (1987) 3864.
[31] P. Zanoni, Chem. Phys. Lipids 32 (1983) 179.
[32] E. Abuin, E.A. Lissi, Prog. React. Kinet. 16 (1991) 1.
[33] E.A. Lissi, E. Abuin, Langmuir 8 (1992) 348.
[34] D. Urry, J. Glickson, D. Mayers, J. Haider, Biochemistry 11 (1972) 487.
[35] A. Campos, E. Abuin, E.A. Lissi, Colloids Surf. A 100 (1995) 155.
[36] D. Rice, E. Olfield, Biochemistry 18 (1979) 3272.
[37] M. Morrow, G. Simatos, R. Srinivasan, N. Grandal, N. Taylor, D. Keough, Biochem. Biophys. Acta 1070 (1991) 209.
[38] P. Jost, O. Griffit, R. Capaldi, G. Vanderkooi, Proc. Natl. Acad. Sci. USA 70 (1973) 480.
[39] D. Chapman, J. Gómez-Fernández, F. Goñi, FEBS Lett. 98 (1979) 211.
[40] C. Stubbs, A. Smith, Biochim. Biophys. Acta 779 (1984) 89.
[41] G. Feigenson, Biochemistry 22 (1983) 3106.
[42] D. Jones, E. Haydon, D. Busath, Biochem. Biophys. Acta 861 (1986) 62.
[43] M. Engelke, P. Bojarski, H. Diehl, A. Kubicki, J. Membr. Biol. 153 (1996) 117.