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# Influence of polymyxins on the structural dynamics of *Escherichia coli* lipid membranes

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**Abstract**

Polymyxins are a family of nonribosomic cationic peptide antibiotics highly effective against Gram-negative bacteria. Two members of this family, Polymyxins B and E (PxB, PxE), form molecular vesicle–vesicle contacts and promote a selective exchange of phospholipids at very low concentrations in the membrane, a biophysical phenomenon that can be the basis of their antibiotic mode of action. To get more insight into the interaction of these antibiotics with the lipid membrane, their effect on the structural dynamics of bilayers prepared with lipids extracted from the membrane of *Escherichia coli* was determined using fluorescently labeled phopholipids. Steady-state anisotropy measurements with probes that localize at different positions in the membrane give information on the effects of polymyxins on the mobility of the phospholipids. Results with PxB, PxE, colymycin M and polymyxin B nonapeptide (PxB-NP), a deacylated derivative with no antibiotic properties, are compared. At low peptide concentrations ( $<2$ mol%) PxB and PxE bind to the membranes superficially, affecting very slightly the ordering of the lipids at the outermost part of the bilayer. Above this concentration, PxB and PxE insert more deeply in the bilayer, increasing lipid order both in the gel and liquid–crystal states and modifying phase transitions. Fluorescence experiments with pyrene labeled phospholipids indicate that the increase in lipid packing is accompanied by an enrichment of phospholipids in the bilayers. In contrast, colymycin M and PxB-NP did not modify lipid packing or phase transition, nor did they induce microdomain formation. The possible significance of these results in the antibiotic mode of action of PxB and PxE is discussed. The combination of spectroscopic techniques described here can be useful as part of a general method of screening for new antibiotics that act on the membrane by the same mechanism as polymyxins.


*Keywords:* Polymyxin; Fluorescence; Lipid vesicles; *Escherichia coli*

*Abbreviations:* DPH, 1,6-diphenylhexa-1,3,5-triene; $I_e$, excimer fluorescence intensity of pyrene at 480 nm; $I_m$, monomer fluorescence intensity of pyrene at 398 nm; MIC, minimum inhibitory concentration; NBD-PE, *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)dioleoylphosphatidylethanolamine; PyrPC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)glycero-*sn*-3-phosphocholine; PyrPG, 1-hexadecanoyl-2-(1-pyrenedecanoyl)glycero-*sn*-3-phosphoglycerol; PxB, polymyxin B; PxE, polymyxin E; PxB-NP, polymyxin B nonapeptide; *r*, anisotropy of fluorescence; SUV, small unilamelar vesicles; TMA-DPH, 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene.

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## 1. Introduction

Cationic peptide antibiotics are ubiquitous in nature [1–4]. The polymyxins, including polymyxin B and polymyxin E (PxB, PxE), are a family of closely related pentabasic peptide antibiotics containing a cycloheptapeptide ring with a C-8 or C-9 fatty acid attached through an amide bond [5]. Polymyxins are nonribosomally synthesized peptides obtained from Gram-positive bacteria, and are rather selective and potent against Gram-negative organisms. Colymycin M, a derivative of PxE with the positive charges neutralized by methane sulfonate, has been developed as an anti-*Pseudomonas aeruginosa* prodrug used in aerosol formulations to treat patients with cystic fibrosis [6]. PxB nonapeptide is a deacylated derivative of PxB with no antibiotic properties; however, it binds to the Gram negative outer membrane with the same affinity as PxB [5]. Although polymyxins were discovered more than 50 years ago, their mode of action is still not precisely known. A number of studies have suggested that polymyxins act on bacterial cytoplasmic membranes [7,8], although most of them were done at high multiples of the MIC. Moreover, the high selectivity of this class of antibiotics precludes that the mechanism of antibiotic action is the permeabilization of the cellular membranes. A new locus for the antibacterial stress by polymyxins has emerged only recently. The basis for the primary antibacterial stress is attributed to the ability of the peptides to form functional contacts between phospholipid interfaces [9]. PxB at low concentration in the membrane promotes a direct, rapid, and selective exchange of phospholipids without (hemi)-fusion, leakage or solubilization of phospholipid vesicles [10,11]. Such observations suggest that the basis for the antimicrobial activity lies in the PxB-mediated contact between the two apposing layers of the inner and outer membranes of the Gram-negative bacteria. This is consistent with the observation that PxB [12] and other cationic peptide antibiotics such as cecropins [13,14] at MIC, induce a highly selective cellular stress that interferes with hyperosmotic response [15] and leads to stasis and cell death. Sublethal concentrations of PxB in growing *Escherichia coli* induce selective transcription of the *osmY* gene without leakage of solutes and protons [14]. Since *osmY* expression is also induced by hyperosmotic stress, it is possible that the loss of phospholipid compositional specificity caused by the PxB-mediated exchange is the origin of the osmotic imbalance that leads to bacteriostasis. This novel antibacterial mechanism has major implications in bacterial resistance, since it would not be susceptible to stable genetic resistance. In this context, even though PxB has been in clinical use for several decades, there is no indication of antibiotic resistance in clinical isolates.

To gain more insight into the molecular basis for polymyxin mode of action in the membrane, in this paper we studied the effect of PxB and PxE on model membranes prepared with lipids extracted from the Gram-negative *E. coli*. Results were compared with the nonantibiotic derivative polymyxin B nonapeptide (PxB-NP) and with the prodrug colymycin M. The effect of different concentrations of polymyxins in the mobility of the phospholipids and the gel-to-liquid crystal phase transition have been studied by steady-state anisotropy, using three types of probes located at different positions in the membrane: 1,6-diphenyl-hexa-1,3,5-triene (DPH), 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH), and *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)dioleoyl phosphatidyl ethanolamine (NBD-PE). Moreover, the possibility of microdomain formation was determined with pyrene-labeled phospholipids.

## 2. Material and methods

### 2.1. Chemicals

Lipid extract from *E. coli* and NBD-PE were from Avanti Polar Lipids (Alabaster, AL); DPH, TMA-DPH, pyrPC and pyrPG were purchased from Molecular Probes (Eugene, OR). PxB, PxE, PxB-NP, and colymycin M (methane sulfonate derivative of PxE) were from Sigma. All chemicals were of the highest available purity.

### 2.2. Lipid vesicles

Unilamelar vesicles alone, with pyrPC or pyrPG, or with the fluorescent probes NBD-PE, DPH, or TMA-DPH, were prepared by evaporation of a mixture of the lipids and probes in $CHCl_3/CH_3OH$ (2:1 v/v). The dried film was hydrated for a final lipid concentration of 20 mM, and then sonicated in a bath type sonicator (Lab Supplies, Hickesville, NY, Model G112SPIT) until a clear dispersion was obtained (typically 2–4 min). Vesicle size was measured by dynamic light scattering with a Malvern II-C autosizer. SUV vesicles have a mean diameter of 80 nm, and a narrow size distribution (polydispersity $< 0.1$).

### 2.3. Measurement of excimer/monomer fluorescence emission

Emission fluorescence from pyrene-labeled lipids was determined in an AB-2 spectrofluorimeter (SLM-Aminco). Vesicles of *E. coli* lipid extract were prepared containing 1% of either pyrPC or pyrPG, and the fluorescence emission was recorded from 360 to 550 nm (excitation 344 nm), with slit-widths of 4 nm for both excitation and emission. Lipid concentration was 133 μM, and samples were magnetically stirred during the experiment. After addition of appropriate amounts of peptides, samples were equilibrated for 2 min before recording the spectra. The emission intensities at 398 and 480 nm were taken for $I_m$ (monomer emission intensity) and $I_e$ (excimer emission intensity), respectively.

### 2.4. Fluorescence anisotropy of membrane lipids

Steady-state anisotropy measurements were conducted in the same instrument used for fluorescence measurements, using L-format fluorescence polarizers. The dynamics of lipids in liposome membranes in the presence of polymyxins were determined by measuring the degree of depolarization of the fluorescence emitted from the probes DPH, TMA-DPH, and NBD-PE. The excitation and emission wavelengths were 365/425 nm for DPH and TMA-DPH, and 460/534 nm for NBD-PE (slit-widths 4 nm). Labeled vesicles with 1% of the desired probe (133 μM lipid) were mixed with the peptide in 10 mM Tris pH 8.0, and anisotropy was measured automatically. For each sample, two cycles were done: a heating cycle from $\sim 5$ up to $\sim 45$ °C, followed by a cooling cycle to the initial temperature, at 1 °C intervals, allowing for thermal equilibration. In some cases, vesicles were titrated with the peptide at a constant temperature, corresponding to a gel or liquid crystal state of the membrane. All solutions were stirred continuously during the measurements. Cuvette temperature was maintained with a Peltier system piloted by a computer program (Microbeam S. A., Barcelona, Spain), and the temperature was registered with a thermocouple inserted into the cuvette. Fluorescence anisotropy ($r$) was calculated automatically by the software provided with the instrument, according to:

$$r = (I_{Vv} - GI_{Vh})/(I_{Vv} + 2GI_{Vh}),$$

where $I_{Vv}$ and $I_{Vh}$ are the intensities of the emitted polarized light with the emission polarizer parallel or perpendicular to the excitation polarizer. Anisotropy values were corrected for dependencies in the detection system ($G$-factor correction, $G = I_{Hv}/I_{Hh}$).

## 3. Results and discussion

### 3.1. Effect of polymyxins on membrane lipid dynamics determined by pyrene fluorescence

Irradiation of *E. coli* lipid vesicles containing 1% pyrene-labeled phospholipids at 344 nm generates a monomeric excited state, which relaxes back to the ground state by emitting photons with a maximum at approximately 398 nm ($I_m$). The contribution from the excimer band ($I_e$, emission at 480 nm), formed by collision between the excited monomer and a ground-state pyrene, is very small at this concentration of pyrene in the membrane. The effect of different concentrations of polymyxins on the dynamics of membrane lipids was assessed by measuring the changes in $I_m$ and $I_e$. The characteristics of the peptides included in this study are listed in Table 1. PxB and PxE are highly active against Gram-negative

Table 1
Structure of the peptides

| Peptide | Sequence and structure[a] | Charge |
|---|---|---|
| Polymyxin B | Cyclic isooctanoyl **BTBB(B**F$^{d}$**LBBT)** | +5 |
| Polymyxin E | Cyclic isooctanoyl **BTBB(B**L$^{d}$**LBBT)** | +5 |
| Polymyxin B nonapeptide | Cyclic T**BB(B**F$^{d}$**LBBT)** | +4 |
| Colymycin M | Methane sulfonate derivative of PxE | 0 |

[a] One-letter aminoacid code. Positively charged residues at neutral pH are shown in bold; B, diaminobutyrate; d, D-enantiomer of the corresponding aminoacid; cyclic aminoacids in parentheses.

bacteria, with MICs of less than 0.25 μM [5,14], whereas colymycin M is ≈30-fold less active [16]. PxB-NP inhibits growth at 200-fold higher concentrations, compared to the parent peptide. Steady-state $I_e/I_m$ values can be correlated with the lateral mobility and the local concentration of the fluorophore in the membrane [17,18]. Two different labeled phospholipids were studied: zwitterionic pyrPC, and anionic pyrPG. The high affinity of cationic polymyxins for acidic phospholipids is well known [2,5,19]. Moreover, we have previously shown that at low concentrations at the interface, PxB forms stable vesicle–vesicle contacts between vesicles of anionic phospholipids, and that there is a fast exchange of phospholipids between the apposed monolayers [9–11]. More importantly, the exchange is selective, so that only monoanionic phospholipids such as PG can cross the PxB-mediated contacts, whereas other components of the membrane, including zwitterionic phospholipids such as PC, are excluded; also, the inner monolayers of the apposed vesicles stay intact, and the aqueous contents do not mix or leak.

Membranes of *E. coli* lipids have a gel-to-liquid crystal phase transition at around 20–25 °C [20], as we confirmed by DSC (not shown), and is in agreement with the anisotropy experiments described later. Experiments were carried out at two temperatures, 15 and 40 °C, with the membrane in a gel or in a fluid phase, respectively. For pyrPG-labeled vesicles at 40 °C, concentrations of PxB and PxE below 1.5 mol% did not modify $I_m$ or $I_e$, whereas a significant and concentration-dependent decrease in $I_m$ was observed at higher concentrations of the peptides. The resulting changes in $I_e/I_m$ (Fig. 1A) clearly show the bi-phasic behavior, with no significant change in ratio at low peptide concentrations, and a progressive increase in the excimer/monomer ratio above ≈2 mol% of the antibiotic peptides in the membrane. For example, at 4 mol% $I_e/I_m$ increased by 13 and 14% for PxB and PxE, respectively. As also shown in Fig. 1, the deacylated derivative PxB-NP as well as colymycin M had no significant effect on pyrPG fluorescence. At 15 °C, PxB and PxE also caused an increase in $I_e/I_m$; although a small effect was observed at low concentrations, the slope of the change was higher above 2 mol% peptide (Fig. 1B). The magnitude of the change was higher than at 40 °C. Colymycin

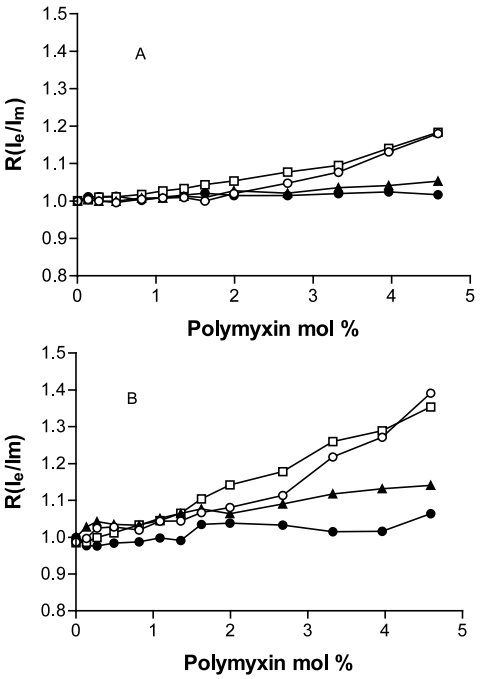


Fig. 1. Effects of different polymyxins on lipid dynamics in *E. coli* unilamelar lipid vesicles assessed by the fluorescence of pyrene-labeled phospholipid pyrPG (1 mol%) shown as changes in the normalized excimer/monomer ratio ($I_e/I_m$). Panel (A): 40 °C; Panel (B): 15 °C. Peptides: PxB (open circles); PxE (open squares); PxB-NP (closed triangles); colymycin M (closed circles). Lipid concentration 133 μM in 10 mM Tris pH 8. Standard deviation from three different experiments is 0.002.

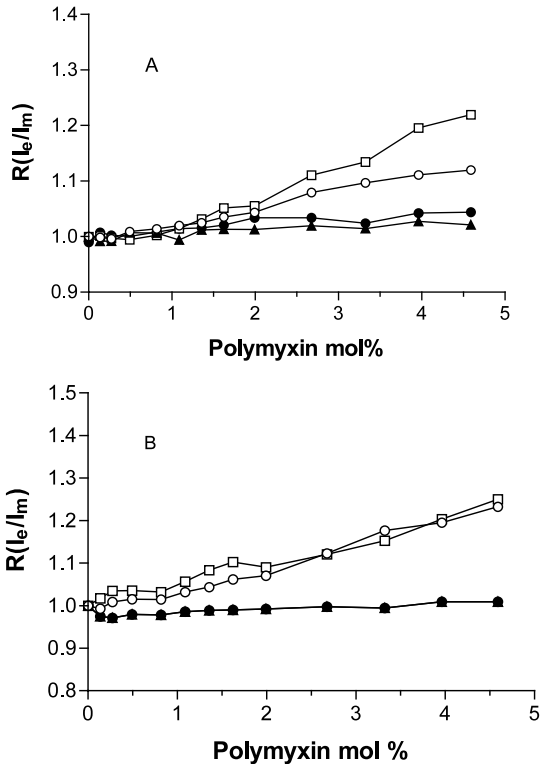


Fig. 2. Effects of different polymyxins on lipid dynamics in *E. coli* unilamelar lipid vesicles assessed by the fluorescence of pyrene-labeled phospholipid pyrPC (1 mol%) shown as changes in the normalized excimer/monomer ratio ($I_e/I_m$). Panel (A): 40 °C; Panel (B): 15 °C. Peptides: PxB (open circle); PxE (open square); PxB-NP (closed triangle); colymycin M (closed circle). Other conditions as in Fig. 1.

M had almost no effect in pyrPG fluorescence, and PxB-NP slightly increased the excimer/monomer ratio.

Results with pyrPC labeled vesicles were very similar (Fig. 2). At 40 °C, PxB and PxE induced a decrement of $I_m$, and a parallel increase in the excimer/monomer ratios at concentrations $>2$ mol% of peptide (Fig. 2A), whereas PxB-NP and colymycin M had no effect in pyrPC fluorescence under these conditions. When the membrane was in the gel phase, at 15 °C, the effect of PxB and PxE in $I_e/I_m$ followed the same trend as described for pyrPG, with a significant increase up to 20% at 4 mol% peptide. The decrement in monomer intensity and small increment in excimer intensity were more important above 1.5 mol% PxB. As also shown in Fig. 2, PxB-NP and colymycin M did not modify pyrPC fluorescence.

The observed increments in $I_e/I_m$ for pyrene-labeled lipids induced by PxB and PxE (Figs. 1 and 2) can result either from lateral segregation of the probe or from an increased rate of lipid lateral diffusion [17,18,21]. To distinguish between these two qualitatively different changes in lipid dynamics, we assessed changes in acyl chain order by measuring fluorescence anisotropy for different probes located in the *E. coli* lipid membrane.

### 3.2. Impact of polymyxins on membrane microviscosity by fluorescence anisotropy

The effect of different concentrations of PxB on the mobility of the phospholipids and the gel-to-liquid crystal phase transition has been studied by steady-state anisotropy, using three types of probes located at different positions in the membrane: DPH, TMA-DPH, and NBD-PE. DPH is a hydrophobic molecule that is oriented predominantly parallel to the fatty acid chains, and the distance of the shallow end of the group to the bilayer center in PC vesicles is around 13 Å [22]. It reports on the order of the lipid fatty acyl chains in the core region of the bilayer. TMA-DPH is anchored at the water/lipid interface, because of its additional charged trimethylamonium group [23], and the distance of the shallow end of the DPH group to the center of PC bilayers is 16.5 Å. Finally, NBD-PE is an indicator of the mobility of the lipid headgroup region of the membranes [24]. Since fluorescence anisotropy is highly dependent upon the viscosity of the solution in which the fluorophore is dissolved, it can be used to obtain information on the microviscosity of lipid membranes where the fluorophore partitions.

The effect of PxB binding on the dynamics of the fatty acyl chains in the hydrophobic core region of the bilayers, reported by the DPH probe, is shown in Figs. 3 and 4. DPH is the most widely used probe for estimating membrane microviscosity. It is a symmetric molecule, and probably has isotropic depolarizing rotations, since the rapid rotations along its long axis are not depolarizing [25]. The phase transitions of membranes are dramatically revealed by DPH, in such a way

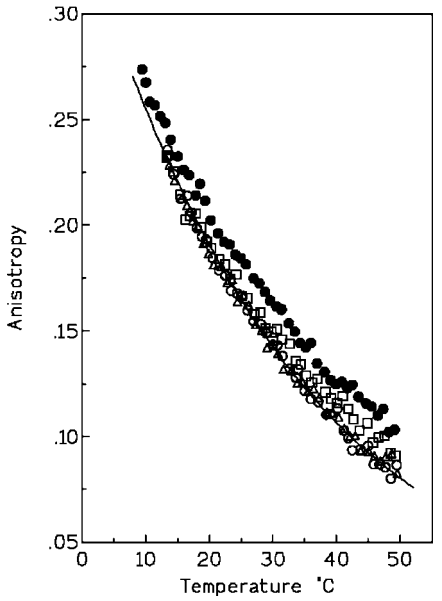


Fig. 3. Temperature dependence of the steady-state fluorescence anisotropy of DPH-labeled vesicles of *E. coli*. Vesicles (133 μM) were cooled in 10 mM Tris pH 8.0 to the starting temperature, and then anisotropies were measured at the desired temperatures. PxB concentration: 0 (open circles and line), 0.5 (open triangles); 2 (open squares); 4 mol% (closed circles). Excitation 365 nm, emission 425 nm. The data are representative of at least three independent experiments (± 0.002).

that it is possible to accurately follow up the lipid phase transition. As shown in Fig. 3, DPH anisotropy in the absence of peptides was relatively high at low temperatures, for example $r = 0.224$ at 15 °C, thus suggesting a quite restricted rotation for the probe. Increasing the temperature resulted in a decrease of anisotropy, due to rotational depolarization as the microviscosity of the membranes decreases, and above 40 °C, anisotropy had very low values ($r < 0.1$). The gel-to-liquid crystal transition in *E. coli* lipid vesicles occurs between 20 and 25 °C, depending on the exact composition and conditions [20]. Even though the transition is not as cooperative as in vesicles of synthetic phospholipids, as expected from a natural extract, the magnitude of the change in DPH anisotropy shown in Fig. 3 clearly indicates that the phase transition has taken place. After a heating cycle, samples were cooled to the initial temperature (not shown), and $r$ values were

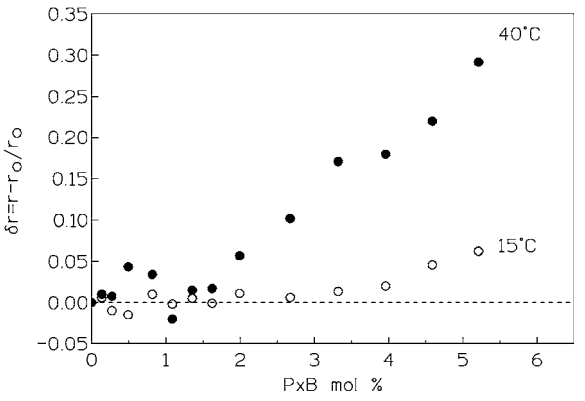


Fig. 4 Steady-state fluorescence anisotropy of DPH in *E. coli* extract lipid vesicles (133 μM) as a function of the concentration of PxB, at constant temperature: 15 (open circles) and 40 °C (closed circles). Other conditions in Fig. 3.

approximately the same (no hysteresis in the liquid crystal-to-gel transition).

The effect of different concentrations of PxB in the phase transition of DPH-labeled vesicles is also shown in Fig. 3. At 0.5 mol% PxB, which corresponds to a lipid–PxB molar ratio or 200:1, a concentration that induces vesicle–vesicle formation and exchange of phospholipids [9–11], PxB did not modify DPH anisotropy in *E. coli* vesicles in all the range of temperatures, from 10 to 50 °C, thus suggesting that the peptide antibiotic does not penetrate in the hydrophobic core of the bilayer. At 2 mol% (lipid–PxB 50:1), a concentration of antibiotic that exhibits membrane-destabilizing properties [10,19], a significant increase in $r$ in the liquid crystal phase of the bilayer ( > 30 °C) was observed. For example, at 40 °C, $r$ increased from 0.103 to 0.118 (results from three different experiments ±0.002). Finally, at high concentrations of PxB in the membrane (4 mol%), when it is known that the peptide induces fusion of membranes with leakage of aqueous contents, anisotropy increases throughout all the temperature range, indicating that the peptide inserts deeply in the bilayer and increases acyl chain order. It is possible that the acyl chain of PxB orients parallel to the acyl chains of the phospholipids, favoring lipid packing. All the depolarization curves (heating cycles) were reproducible in the cooling cycles (not shown for clarity), indicating that the peptide

is tightly bound to the interface. A similar behavior has been recently described for the cationic antibiotics magainin and indolicin with vesicles of acidic phospholipids [21]. The bi-phasic profile of the DPH anisotropy change as a function of PxB concentration is shown in Fig. 4. DPH anisotropy increased significantly above 2 mol% PxB, and the increase in lipid packing was more important when the membrane was in the fluid phase, at 40 °C. PxE-induced changes in acyl chain mobility were essentially the same as described for PxB, whereas PxB-NP and colymycin M had no effect on lipid packing in the hydrophobic core of the membrane, independently of the concentration of peptide added (not shown).

Lipid mobility was reduced in the region closest to the glycerol backbone of the *E. coli* membranes, as deduced from the higher $r$ values of the TMA-DPH probe compared to $r$ values for DPH at the same temperatures (Fig. 5). Binding of PxB at low concentrations did not modify lipid order in this region of the membrane, indicating that the peptide does not penetrate at this level of the bilayer. However, at concentrations of 2 mol%, and more importantly at 4 mol%, a PxB-induced decrease in chain mobility was observed (Fig. 5). This increase in chain order was observed both in the gel and in the liquid crystal states of the lipid, and is consistent with a strong insertion of the peptide at the interfacial region of the membrane. Experiments at a fixed temperature showed that the rigidifying effect depended on the peptide concentration (Fig. 6A). Upon increasing PxB

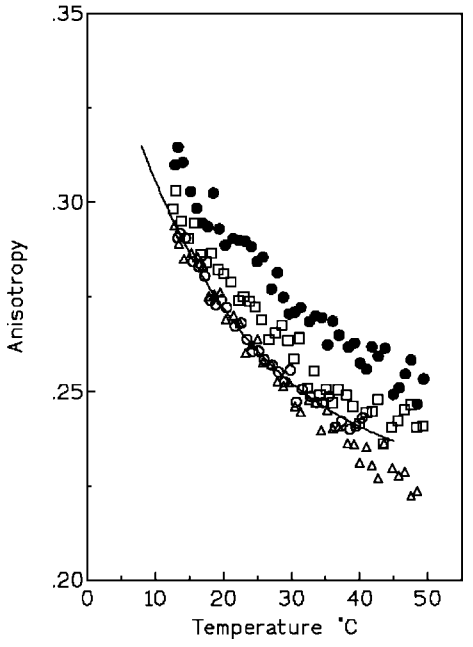


Fig. 5. Temperature dependence of the steady-state fluorescence anisotropy of TMA-DPH-labeled vesicles of *E. coli*. Vesicles (133 μM) were cooled in 10 mM Tris pH 8.0 to the starting temperature, and then anisotropies were measured at the desired temperatures. PxB concentration: 0 (open circles and line), 0.5 (open triangles); 2 (open squares); 4 mol% (closed circles). Excitation 365 nm, emission 425 nm. The data are representative of at least three independent experiments (± 0.002).

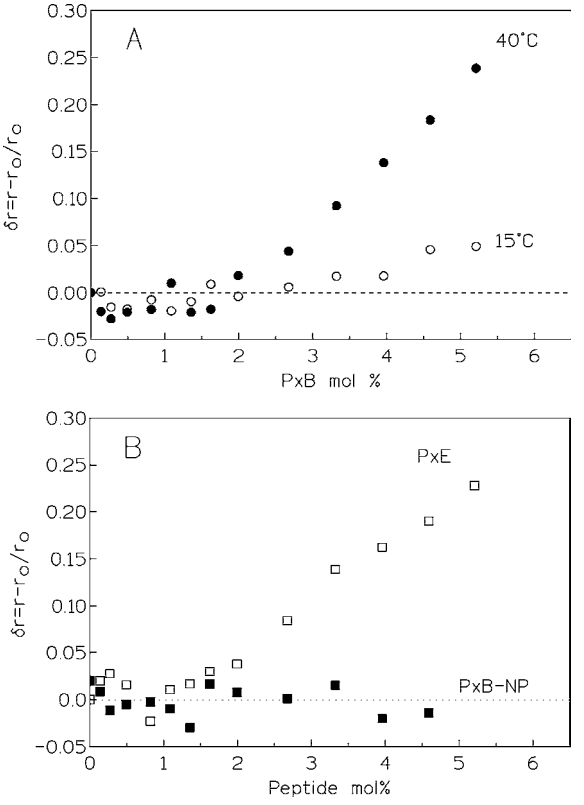


Fig. 6. (A) Steady-state fluorescence anisotropy of TMA-DPH in *E. coli* extract lipid vesicles (133 μM) as a function of the concentration of PxB, at constant temperature: 15 (open circles) and 40 °C (closed circles). (B) Same for PxE (open squares) and PxB-NP (closed triangles) at 40 °C. Other conditions as in Fig. 5.

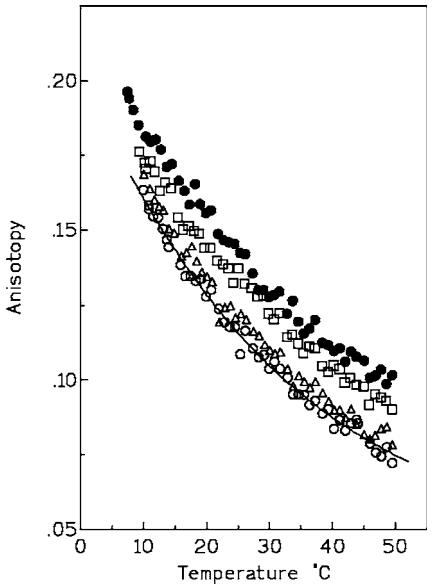


Fig. 7. Temperature dependence of the steady-state fluorescence anisotropy of NBD-PE-labeled vesicles of *E. coli*. Vesicles (133 μM) were cooled in 10 mM Tris pH 8.0 to the starting temperature, and then anisotropies were measured at the desired temperatures. PxB concentration: 0 (open circles and line), 0.5 (open triangles); 2 (open squares); 4 mol% (closed circles). Excitation 460 nm, emission 534 nm. The data are representative of at least three independent experiments (± 0.002).

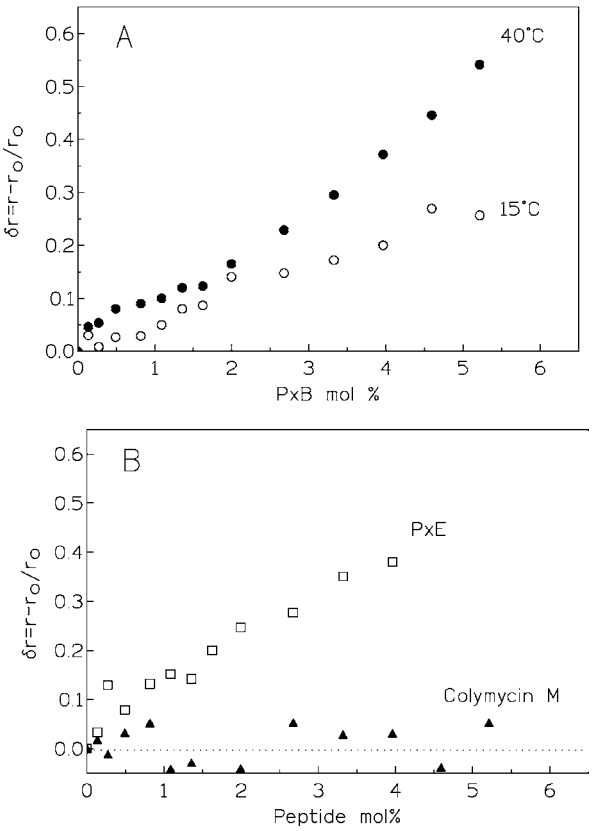


Fig. 8. (A) Steady-state fluorescence anisotropy of NBD-PE in *E. coli* extract lipid vesicles (133 μM) as a function of the concentration of PxB, at constant temperature: 15 (open circles) and 40 °C (closed circles). (B) Same for PxE (open squares) and colymycin M (closed triangles) at 40 °C. Other conditions as in Fig. 7.

concentration up to ≈2 mol% in the membrane, TMA-DPH anisotropy did not change significantly. Following this initial phase, there was a second process characterized by a linear increase in anisotropy with PxB concentration. PxE had the same effect than PxB in the *E. coli* lipid vesicles (Fig. 6B), whereas in the presence of PxB-NP and colymycin M there was no change in anisotropy.

Lipid mobility in the headgroup region of the *E. coli* membrane was high, as deduced from the low *r* values of NBD-PE (Fig. 7). Binding of PxB strongly decreased the mobility at the headgroup region of the vesicles, both in the fluid and, more significantly, in the crystal liquid phases, as deduced from the increase of NBD anisotropy. Interestingly, the increment in lipid order in this external region was observed at very low concentrations of PxB, starting at <0.2 mol% (Fig. 8A). The increase in anisotropy had two slopes, with significantly higher increments at PxB concentrations above 2 mol%. PxE had the same effect as PxB, for example the anisotropy change as a function of concentration at 40 °C is shown in Fig. 8B. These results suggest that PxB and PxE strongly interact with the headgroup region of the *E. coli* vesicles, decreasing lipid mobility. PxB-NP, as well as colymycin M, showed no significant effect. Cooling cycles did not have significant hysteresis, even at 4 mol% PxB (not shown), indicating that the peptide did not leave the interface when the lipids adopt the more compact crystalline state.

## 4. Conclusions

Polymyxins are a family of cationic peptide antibiotics that target the Gram-negative membranes. Although their mechanism of action remains to be elucidated in detail, biophysical studies using model membranes of synthetic phospholipids have demonstrated that at low concentrations of PxB and PxE in the membrane, below 2 mol%, the peptide forms vesicle–vesicle contacts between vesicles of anionic phospholipids and induces phospholipid exchange [9–11]. In this paper we studied the effects of different polymyxins and polymyxin derivatives in the physicochemical properties of lipid membranes formed by natural lipids of *E. coli*, a prototype of Gram-negative bacteria. Results are consistent with the existence of two different forms of PxB at the interface, as also concluded previously from circular dichroism and fluorescence experiments in vesicles of synthetic phospholipids [11] and from mono- and bidimensional NMR experiments combined with simulated annealing calculations [26]. At low concentrations of the peptide in the membrane, below 2 mol% PxB (lipid–PxB 50:1 mol/mol), a range in which PxB forms vesicle–vesicle contacts and exchange of phospholipids and is in the order of the MIC for this antibiotic, PxB binds to vesicles prepared with natural lipids of *E. coli* and inserts in the external part of the membrane. Excimer/monomer pyrene fluorescence experiments together with anisotropy data using external, interfacial, and internal probes indicate that the form of PxB in the vesicle–vesicle contacts remains in the outermost part of the membrane and has no significant effect in membrane microviscosity, lateral diffusion or microdomain formation. Only a small decrement of lipid dynamics was observed in the outer part of the membrane, where the peptide is bound. The same is true for PxE, another member of the polymyxins family with the same antibiotic and contact-forming properties as PxB. On the other hand, at concentrations above 2 mol%, PxB and PxE adopt a different form at the interface, with destabilizing properties. This form of the peptides is bound to the anionic interface but not in an intervesicle contact, and has been characterized previously [19]. PxB $>2$ mol% in anionic vesicles [11], and in vesicles of *E. coli* lipids [27] promotes membrane fusion accompanied by nonspecific lipid mixing and leakage of aqueous contents. Under these conditions, PxB and PxE have a strong effect on the phase properties of the membrane. The peptides insert deeply in the bilayer, increasing lipid packing; this can be explained by the insertion of the acyl chain of the polymyxins that will be parallel to the fatty acids of the phospholipids, favoring lipid order. This will explain why the deacylated derivative PxB-NP had no effect on membrane microviscosity. The neutral lipopeptide colymycin M had no effect on lipid dynamics, due to the absence of the necessary cationic charges. This is consistent with previous studies on the interaction of colymycin M with model membranes [16]. Probably, as suggested by other authors, colistin methane sulfonate acts as a slow-release prodrug for PxE. To summarize, we present a combination of two methods based on fluorescence spectroscopy that provide information on the influence of peptide antibiotics on the microviscosity, fluidity and molecular mobility of membrane lipids. Results presented here are consistent with the current hypothesis that polymyxin-mediated cell death is not due to the alteration of membrane permeability, an event that takes place at high concentrations of the peptide, of several-fold the MIC. At the MIC, polymyxin also acts on the bacterial membrane, but by a different mechanism that probably involves lipid scrambling between the phospholipid interfaces surrounding the periplasmic space [12–15]. The experiments described here together with previously described protocols to detect selective vesicle–vesicle lipid exchange [9–11] can be a general method of screening for new antibiotics that act in the membrane by the same mechanism as polymyxins.

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