International Journal of Pharmaceutics 160 (1998) 99–107

Interaction of colistin with lipids in liposomes and monolayers

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Received 6 June 1997; received in revised form 10 September 1997; accepted 12 September 1997

Abstract

Colistin is an antibiotic member of the polymixin family, showing a high amphipathic character. Its mechanism of action has been related to its ability to disrupt phospholipid bilayers of the bacterial membrane. Due to its hydrophobic properties colistin can interact both with the polar heads and the alkyl chains of the phospholipids. Moreover, it has a polycationic structure, so its interactions with phospholipids should be highly dependent on the electric charge of the lipid and the ionisation state of polymixin molecule. In the present paper we report on physicochemical studies to define the type and characteristics of these interactions. The surface activity of colistin has been shown to be highly dependent on the pH of the medium, the less protonated forms being more stable at the air–water interface. Interaction with phospholipid monolayers shows the same tendency. Colistin is also able to form mixed monolayers with phospholipids with small deviations from ideality. Physicochemical studies carried out with fluorescent probes indicate the presence of pure colistin aggregates that can coexist with mixed micelles composed of colistin/phospholipids. In no case did the interaction cause significative changes in the transition temperature of phospholipids. © 1998 Elsevier Science B.V.

Keywords: Monolayers; Polarisation fluorescence; Liposomes; DPPC; DPPG; Colistin

1. Introduction

Colistin is a polycationic antibiotic acting at the membrane level. It has been suggested that the antibactericidal activity of polymixin E (colistin), is mainly due to its ability to disorganise cytoplasmatic membranes of susceptible bacteria, through electrostatic interaction with its components. This behaviour is associated with a permeability change, that alters the osmotic integrity of the cell structure, due to the amphipaticity of the colistin molecule (Pratt, 1977; Brown and Wood, 1972).

Colistin possesses a charged cyclic heptapeptide head group with a tripeptide tail to which a fatty

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PII S0378-5173(97)00301-3

acid residue is attached (Fig. 1). The presence of the peptide cationic moiety seems to account,at least in part, for the ability of this molecule to bind to certain negatively charged phospholipids in the membrane of pathogen microorganisms (Sebek, 1967).

It has been shown (Moore et al., 1980), for instance, that polymixin B interacts with the lipopolysacharide (LPS) and Lipid A from *Pseudomonas aeruginosa*. This binding process was displaced by divalent cations and other aminoglycosides, thus indicating an important contribution of electrostatic forces.

Moreover, the actions of polymixins on membranes have been studied using liposomes as a model system and it has been suggested that negatively charged amphipathic molecules such as phosphatidylglycerol or cardiolipin are required for sensitivity to polymixins (Shirai and Aida, 1984).

It seems that both electrostatic and hydrophobic properties contribute to the interaction of polymixins with biomembranes.

In this paper we report a physicochemical study of the interaction of colistin with neutral and negatively charged phospholipids of different alkyl chain length. Moreover, a tendency to self aggregation of colistin in aqueous solutions is also described, as well as its surface activity and ability to form monomolecular layers.

The results obtained indicate that colistin forms stable aggregates in aqueous solution that interact very slowly and weakly with mono and bilayers of phospholipids.

2. Materials and methods

2.1. Chemicals

Dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine (DSPC), dipalmitoyl phosphatidylglycerol (DPPG), 8-anilinonaphtalene sulphonate (ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were from Sigma. Colistin (Polymixin E) was a gift of Infavet S.A.

Fluorescence polarisation experiments were carried out in sodium acetate solution (TAC) at pH 7.4.

2.2. Methods

2.2.1. Surface activity

Surface actitivity was measured by injecting different volumes of a colistin concentrate solution into an aqueous subphase (PBS, pH 7.4) and recording the surface pressure increases. The teflon trough had 70 ml capacity and the subphase was continuously stirred.

2.2.2. Compression isotherms

Compression isotherms were recorded on a Langmuir film balance equipped with a Wilhelmy platinum plate, as described by (Verger and De

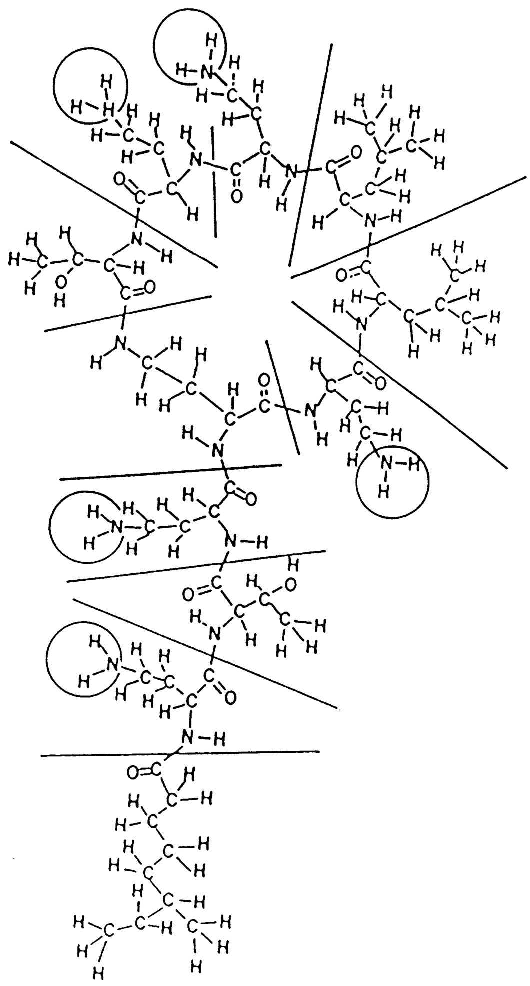

Fig. 1. Colistin structure.

Haas, 1973). The output of the pressure pickup (Sartorius microbalance) was calibrated by recording the well-known isotherm of stearic acid. This isotherm is characterised by a sharp phase transition at 25 mN/m in pure water at 20°C. The teflon trough (surface area: 498 cm^2, volume: 330 ml) was regularly cleaned with hot chromic acid and rinsed with double distilled water.

Chloroform solutions of the lipids were spread on the aqueous surface and 10 min were allowed for solvent evaporation. Films were compressed at a rate of 4.2 cm/min. All the isotherms were run at least four times in the direction of increasing pressure with freshly prepared films. Stability of the monolayers was assessed by compressing monolayers to a pressure of 25 mN/m, stopping the barrier and observing the pressure decay. No pressure changes were observed after 30 min. All the measurements were made at a surface temperature of 21 ± 1°C.

Sodium chloride solutions 0.9% with or without colistin (1.7×10^{-6} M), were used as subphases and adjusted to different pH values.

Standard deviation for isotherms was less than 1%.

2.2.3. Insertion of colistin into monolayers

Stock solutions of lipids were prepared in chloroform, and different volumes of these solutions at a concentration approximately of 1 mg/ml were spread at the air–water interface using a Hamilton syringe, to attain 5, 10 or 20 mN/m of surface pressure. After a stabilisation period of 10 min, colistin was injected into a continuously stirred subphase in small volumes from a concentrate aqueous solution. The equilibrium value for surface pressure increases was determined after 60 min of injection. Maximum standard deviation in the assays was 0.8%.

2.2.4. Membrane fluidity

Small vesicles were prepared by probe sonication of a lipid dispersion in acetate solution, followed by ultracentrifugation at 27 000 rpm for 40 min, all the processes being carried out at temperatures higher than that of lipids transition. Sizes ranged between 120 and 140 nm in diameter (measured in a Malvern nanosizer). Phospholipid phosphorous concentration was determined using the Bartlett method (Bartlett, 1959).

Aliquots of vesicles were incubated with ANS and DPH at several phospholipids/probe relationships for 60 min in order to achieve saturation level.

Liposomes saturated with these fluorescent probes were incubated with different amounts of colistin, and the fluorescence polarisation measured as a function of the temperature. Excitation and emission wavelengths were 398 and 480 nm for ANS and 357 and 450 nm for DPH.

Experiments were carried out on a Perkin-Elmer spectrofluorimeter LS 50 equipped with thermostated cuvettes and polarizers.

3. Results and discussion

3.1. Surface activity

The surface activity of colistin was determined for different concentrations of this molecule in the subphase, working at pH 7.4. The pressure increases obtained reflect the incorporation of this molecule to the surface and allow us to calculate the surface excess (G) of colistin by applying the Gibbs Eq. (1):

$$\Gamma = \frac{1}{RT} \times \frac{\Delta\pi}{\Delta \ln c} \quad (1)$$

where $\Delta\pi$ is surface pressure increase and c is the concentration of colistin in the subphase. Values of $\Delta\pi$ and Γ are represented in Fig. 2. From the surface pressure increases it can be seen that the molecule has a high surface activity, as was to be expected from the structure given in Fig. 1.

Due to the cationic nature of colistin it seems logical to think that the pH of the media will have a strong influence in its hydrophobicity, increasing this parameter as the number of protonated amino groups decrease. For this reason the pH profile of surface pressure increases was determined and is given in Fig. 3. Colistin concentration in the subphase was 1.7×10^{-6} M.

A similar behaviour was described for Dibucaine a basic molecule susceptible to be protonated in different extension depending on the pH of the media (Seelig, 1987).

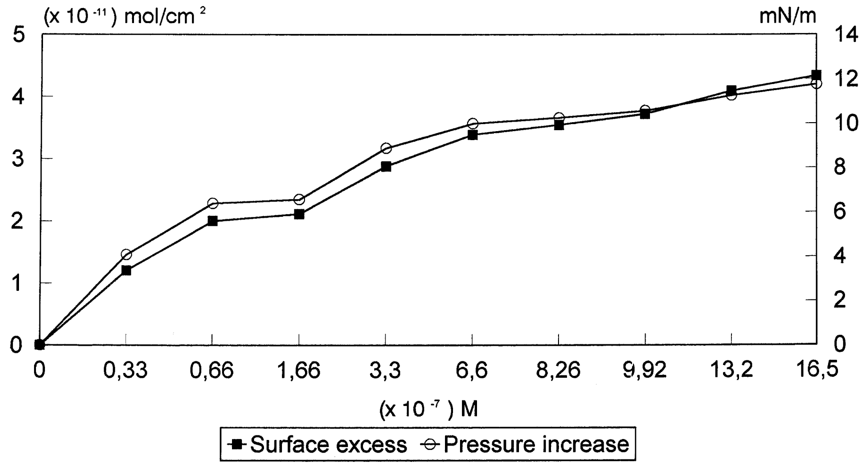

Fig. 2. Surface excess of colistin and pressure increases represented as function of the concentration of colistin in the bulk phase.

It should be noted that there is a direct relationship between pH and surface activity at low solute concentrations and the tendency towards saturation as this variable increases pH. It seems to point to the beginning of an aggregation process due to the total neutralisation of protonated aminogroups.

A similar behaviour was found with hydrophobic peptides (Bogdam et al., 1994; Fonseca et al., 1993).

3.2. Monolayer studies

3.2.1. Measurements at constant surface area

DPPC and DSPC are neutral phospholipids showing a high transition temperature, good stability and tigthly packed bilayers. In addition, as colistin is a polycation, the use of a negatively charged phospholipid, DPPG, seemed interesting to check for electrostatic interactions.

The ability of colistin molecules to interact with phospholipid monolayers was measured through the pressure increases generated in monolayers spread at different surface pressures, after injection of colistin into the subphase.

The antibiotic concentration in the aqueous bulk phase was initially 1.7×10^{-6} M. Phospholipid monolayers were spread at 5, 10 and 20 mN/m of initial pressure, and subphase pH were 1.6, 7.4 and 8.0.

The results are given in Table 1. According to its chemical structure, and to the values represented in Fig. 3, colistin surface activity at basic pH, as a consequence shows a higher tendency to be incorporated into monolayers.

Moreover at low pressures, this behaviour seems to be almost independent on the electric charge of monolayers. Only at very acidic pH (1.6) when all aminoacid groups are protonated, can a weak interaction with DPPG monolayers be seen at 20 mN/m, while no interaction at all is observed for DSPC or DPPC monolayers.

Values reported in Table 1 indicate that colistin interacts with all the phospholipids and the pressure increases are dependent on initial surface pressure. According to the data described above colistin penetrates less strongly in DPPC monolayers, than in DPPG or DSPC. We expected a stronger interaction with DPPG (that has a negative charge), due to electrostatic forces, than with DPPC or DSPC (neutral phospholipids). Considering the shape and slope of DSPC and DPPC compression isotherms, no significant differences on compressibility could be detected, that could explain different levels of penetration found experimentally. There are in the literature references (Cajal et al., 1996; Jain et al., 1982) to the presence of defects in bilayers due to the lack of a good readjustment of phospholipids alkyl chains during liposomes preparation. These defect points

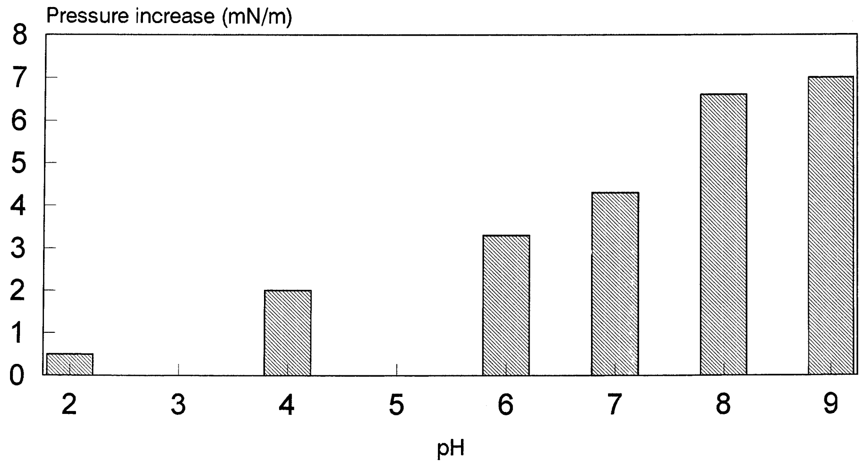

Fig. 3. Dependence of surface activity on pH values for colistin aqueous solutions. Colistin concentration in the subphase: 1.7×10^{-6} M.

will favour the interaction of other molecules with bilayers. In the present situation as DSPC has a higher transition temperature than DPPC, the probability of defects should be higher and in consequence by analogy with bilayers this fact could favour the interaction of colistin with DSPC monolayers.

3.2.2. Compression isotherms of colistin

As colistin showed a high surface activity we checked the ability of this molecule to form stable monolayers when spread on aqueous subphases of different pH values (data not shown). Chloroform/MeOH was used as spreading solvent, and subphases were sodium chloride 0.9% solutions with a pH that had been adjusted to 2, 4, 5.5, 6.5, 7.4 and 8.5.

The shape and the slope of the isotherms were indicative of low stability of the monolayers. Thus suggesting a solubilization process during compression. Area/molecule values measured at 5 mN/m varied from 0.19 to 0.54 nm^2 for pH 2–8.5. Moreover, maximum surface pressures achieved were in all cases lower than 12 mN/m.

These differences in area/molecule could be due either to a solubilization process or to changes in the ordered state of the molecules produced by the compression process.

Based on data already published (Prass et al., 1987) the area/molecule for a densely packed monolayer of a single alkyl chain compound is around 0.8 nm^2, its not possible to imagine that the cross sectional area of colistin should be so small, unless it forms several layers or if it suffers solubilization.

As the shape and slope of its isotherm do not present any transition indicative of any kind of change, we consider that a solubilization process is probably the best explanation for these results.

3.2.3. Compression isotherms of DPPC, DSPC and DPPG on colistin containing subphases

The interaction between colistin and DPSC, DPPC or DPPG was also studied in a another model by spreading phospholipid monolayers on colistin/phosphate buffer solution (PBS) adjusted at pH 7.4 and carrying out the compression isotherms. Comparing the shape and the area/molecule values of these isotherms, an expansive effect can be clearly seen all over the compression process.

At low initial pressures (0.1 mN/m) the molecules of colistin in the bulk of the solution can be incorporated freely to the surface as in the absence of monolayer. Moreover, this penetration modifies the ordered state of phospholipid molecules in the monolayer, as can be appreciated from the pressure area curves obtained on compression, (Fig. 4). The main characteristics are that, DPPC, DSPC and DPPG, monolayers are

Table 1
Pressure increases in lipid monolayers induced by the presence of colistin (bulk phase concentration 1.7 μM) in the subphase (measured at three pH values)

	$\Delta\pi$ (mN/m)								
	pH 1.6			pH 7.4			pH 8		
P_i (mN/m)	5	10	20	5	10	20	5	10	20
DSPC	4.3	2.3	0	8.8	6.7	3.1	12.9	10.1	3.3
DPPC	2.4	2.3	0	5.1	3.8	1.1	11.0	7.2	1.6
DPPG	3.3	1.9	1.9	5.5	4.4	2.7	11.5	7.2	4.5

more expanded than those of the phospholipid spread on PBS, specially at low surface pressures, and DPPC transition phase disappears. Moreover, colistin remains incorporated into the monolayers of DPPC and DSPC during the compression process giving an area/molecule increase of 0.1 or 0.08 nm^2 at 50 mN/m. In contrast, for DPPG monolayers (not shown), this expansion effect is highly dependent on the surface pressure. Similar results have been described between apocytochrome *c* and DOPS/DOPC monolayers (Pilon et al., 1980). This low level of interaction with DPPG is in agreement with the results obtained from penetration experiments, and as several authors suggest can be due to the existence of a strong potential barrier in DPPG monolayers. But, it is also possible that positively charged colistin associates with phosphatidyl glycerol molecules to form some type of micelles or aggregates that on compression can move towards the aqueous phase in form of stable mixed micelles. This phenomenon could be considered as a partial solubilization of phospholipid molecules through its association with colistin.

3.2.4. Mixed monolayers

The miscibility of DPPC, DSPC and DPPG with colistin in monolayers was studied for different molar compositions. Mixed monolayers were prepared from chloroform solutions of both colistin and phospholipid with the same molarity. In this set of experiments the ionic strength and the pH of the subphase were varied in order to determine the influence of these factors in the miscibility pattern of phospholipid–antibiotic molecules. The subphases assayed were NaCl 0.9% pH 5 and 4; PBS pH 7.4 and water pH 5.

In general the presence of colistin in the mixture has no effect on the shape of the isotherms but there is a gradual decrease in the area/molecule values. This behaviour is illustrated in Fig. 5, for DSPC/colistin mixtures spread on PBS, at pH 7.4 subphase.

The area/molecule, measured at 4 mN/m of surface pressure, and plotted as a function of the molar fraction is given in Fig. 6 for DPPC/colistin mixtures.

Deviations from ideality are important for colistin/DPPC mixtures spread on acidic solutions. In contrast, at pH 7.4 colistin electrical charges are almost neutralised and the molecule behaves more like a lipid than as a peptide and the mixtures behave ideally at all the molar compositions. This behaviour, can be due to the fact that protonated colistin molecules exhibit intermolecular repulsive electrostatic forces in the monolayer that result in high values of area/molecule, compared with the low level interactions present in neutral or slightly ionised molecules. Due to the low compression pressure achieved with pure colistin monolayers it was not possible to calculate the energies associated with these deviations from ideality.

3.3. Membrane fluidity

Two fluorescent probes, ANS and DPH, were used to study the effect of colistin on the microviscosity of bilayers, as well as its own tendency to form micelles. The polarisation fluorescence of these two molecules gives information about the motion of the polar heads and alkylchains of phospholipid bilayers, respectively.

As colistin has an amphipathic structure the interaction of this molecule with both markers was previously determined in order to detect some type of colistin aggregates or micelles in aqueous solution. Both ANS and DPH do not show fluorescence in aqueous media unless the molecules are buried in a hydrophobic environment.

The results showed that ANS and DPH after incubation with colistin gave a finite moderate fluorescence intensity and a low value of polarisation with a weak temperature dependence ranging

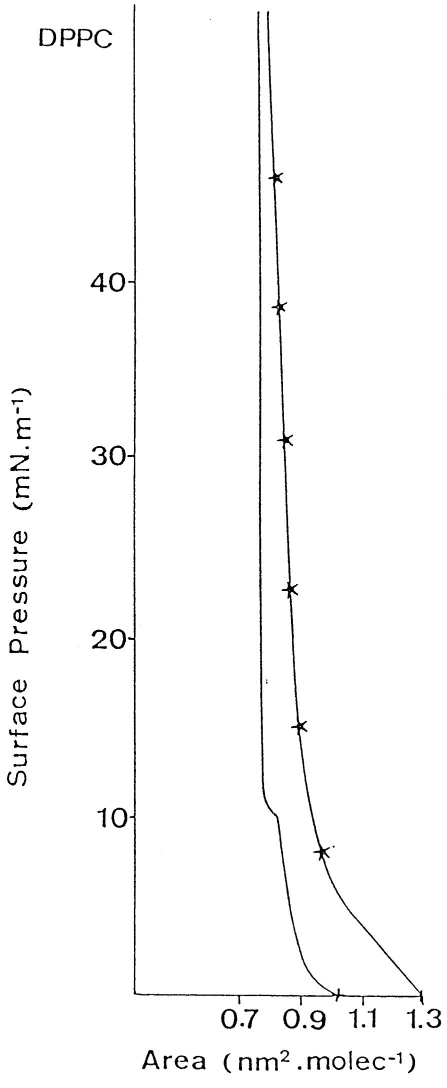

Fig. 4. Compression isotherms of DPPC monolayers spread on PBS pH 7.4, and on colistin 1.7×10^{-6} M solution.

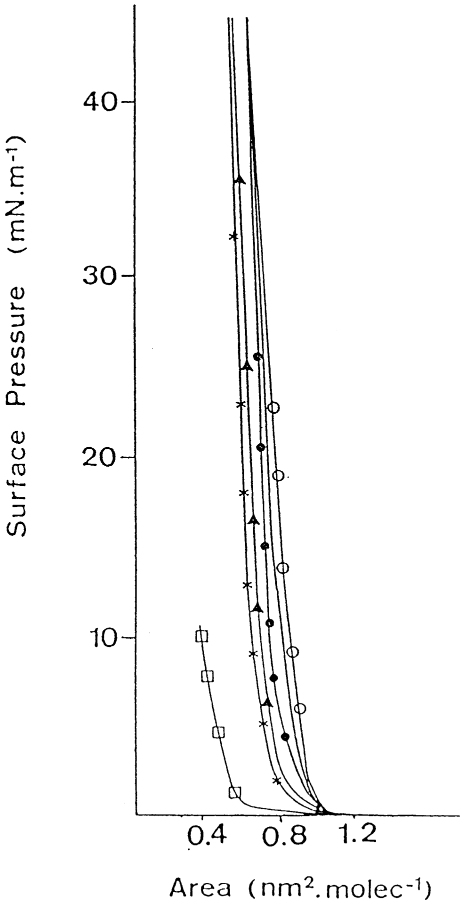

Fig. 5. Mixed monolayers of DSPC and colistin. Subphase PBS pH 7.4. – □ –, colistin; – x –, DSPC 0.2/Col 0.8; – ▲ –, DSPC 0.4/Col 0.6; – • –, DSPC 0.6/Col 0.4; ——, DSPC 0.8/Col 0.2; – ○ –, DSPC.

between 0.168–0.110 and 0.167–0.131 for ANS and DPH, respectively, (measurements made from 20 to 55°C). As indicated above, these results can be due to the entrapment of ANS or DPH molecules in colistin micelles or aggregates, but in the case of ANS the formation of some type of salt in which ANS and colistin molecules will remain associated by electrostatic links can not be discarded. In this case, these pair-ions would move freely in solution and this will explain the low polarisation values detected. Fig. 7(a)–(c) shows changes detected in the polarisation fluorescence of DPH/liposomes represented as a function of temperature for different colistin concentrations and liposomal compositions.

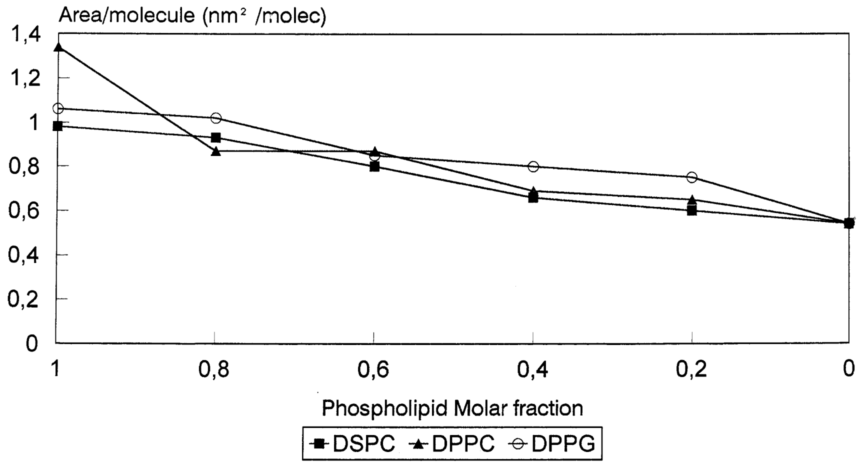

Fig. 6. Area/molecule as function of the molecular fraction of DPPC measured at 4 mN/m and 10 mN/m.

The presence of colistin in the media has a strong fluidifiying effect on bilayer core, as can be seen in all cases by an important decrease in the polarisation values. Nevertheless, when applying the mathematical treatment to these values by adjusting them to a sigmoid curve (Implot computer program), transition temperatures obtained are similar and the confidence intervals lie in the same range, thus indicating that differences among them are not significant.

Moreover, the effect is comparatively low considering interactions detected with other hydrophobic compounds in which phospholipids (PL)/peptide relationships are two or three orders of magnitude higher. Besides, no special differences were found due to the opposite electrical charges of PG and colistin. These results are in agreement with those published in a previous paper (Colomé et al., 1993) in the sense that colistin interaction with phospholipids is mainly hydrophobic.

This behaviour can be due to the formation of stable colistin micelles that leave a very small percentage of free colistin molecules in a solution to be insert into the phospholipid bilayers.

Only with a high excess of colistin in relation to phospholipids does the interaction lead to distortion of the ordered state of bilayers. The existence and stability of micelles is also in agreement with the low values of $\Delta\pi$ represented in Fig. 2.

The use of ANS as a fluorescent marker is complementary due to this interaction because it is located at the external part of the bilayer. The presence of colistin in the media containing DSPC and DPPC liposomes saturated with ANS reduces the polarisation values of these markers, thus indicating an increase in the motion of phospholipids polar head groups (Fig. 8). However, this effect decreases with temperature, suggesting a rigidification of bilayers at high temperatures.

It is not easy to interpret this inversion in the tendency but the possibility of formation of mixed micelles with a new geometry can not be discarded, specially having in mind the almost ideal miscibility behaviour determined when working with monolayers.

Liposomes composed of DPPC-DPPG showed initially low levels of incorporation of ANS giving, as a consequence, weak fluorescence bands and a great fluctuation in polarisation values. For this reason the study of this system was discarded.

As a conclusion, it seems that colistin shows a great tendency to form micelles in aqueous media; these aggregates are stable and interact very weakly with mono and bilayers of phospholipids in drastic conditions. This interaction can be either by insertion between the alkyl chains or through the formation of mixed micelles.

Acknowledgements

This work was supported by a grant BIO-095-0061-03-02 from CICYT (Comisión Asesora de

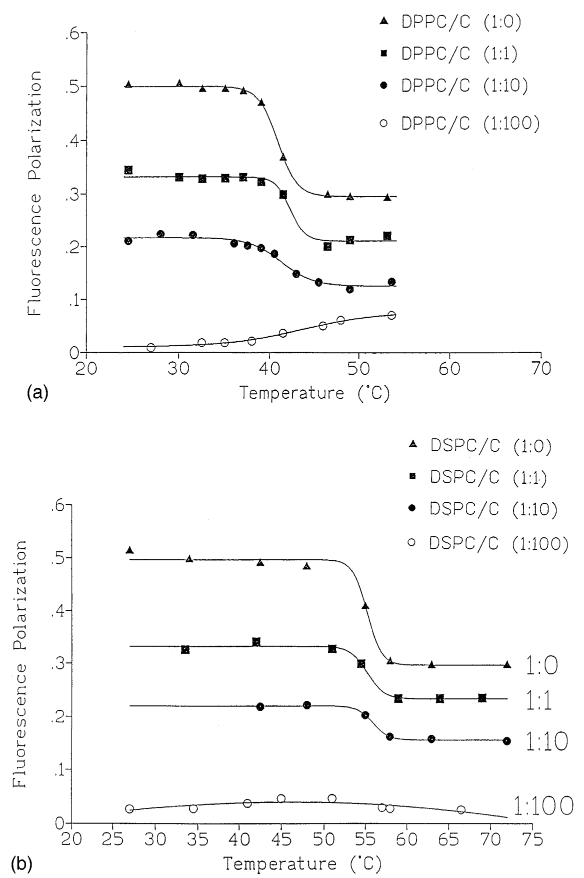

Fig. 7. Polarisation fluorescence variation as function of the temperature in liposomes of (a) DPPC-colistin-DPH, (b) DSPC-Cclistin-DPH and (c) DPPC/DPPG-colistin-DPH, for different molar relations of phosophilips (PL)/colistin, (C).

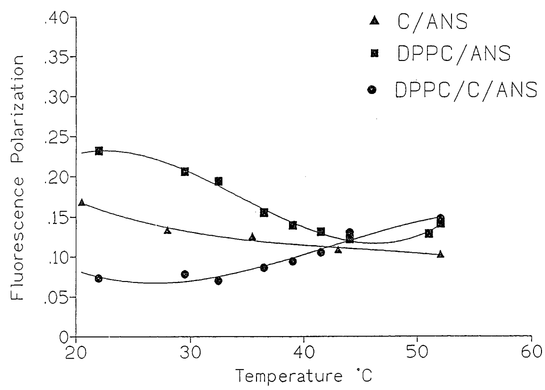

Fig. 8. Polarisation variation produced by colistin when added to ANS–DPPC liposomes.

Investigación Científica y Técnica del Ministerio de Educación y Ciéncia) Spain. We acknowledge the technical assistance of M. Sanchez.

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