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International Journal of Pharmaceutics 252 (2003) 149–157
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Does ciprofloxacin interact with neutral bilayers? An aspect related to its antimicrobial activity

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Received 14 June 2002; received in revised form 1 November 2002; accepted 18 November 2002

Abstract

Ciprofloxacin (CPX) physicochemical properties, mainly hydrophobicity and microspeciation, appear to be related with the ability of this drug to adsorb and diffuse through lipid environments. We have combined the information from fluorescence anisotropy, quenching and epifluorescence of phospholipid monolayers, to explore effects of CPX at the phospholipid-buffer interface. Two fluorescent probes (TMA-DPH and PA-DPH) located at surface level were used for anisotropy experiments. The results evidenced that CPX interact with liposomes at surface level and induce a moderate decrease in the bilayer anisotropy. By using two hydrophobic quenchers (iodobenzene and iododecanoic acid) the presence of CPX in the core of the bilayer was excluded. Mixed monolayers of DPPC and CPX evidenced the ability of CPX to compress the monolayer and the epifluorescence observations showed that CPX modifies lipid distribution and surface phase transition. The surface activity of CPX is reviewed from the physicochemical properties of the drug and in relation to its pharmacological activity.

Keywords: 6-Fluoroquinolones; Membranes; Monolayers; Fluorescence; Anisotropy

1. Introduction

Quinolones are a group of antibacterial agents derived by systematic modification of the nalidixic acid. Among them 6-fluoroquinolones (6-FQs), having a fluorine substituent at the C-6 position have been the focus of research in many fields because of their therapeutical efficiency. The physicochemical properties of 6-FQs have been widely studied: (i) solubility (Ross and Riley, 1990), (ii) ionization–structure relationships (Ross and Riley, 1992), (iii) microspeciation (Takács-Novák et al., 1990), (iv) partitioning using the octanol-buffer model (Ross et al., 1992; Takács-Novák et al., 1995; Fallati et al., 1996, Vázquez et al., 2001a), (v) complexation (Ross and Riley, 1993a,b), (vi) structure–activity relationships (Chu and Fernandes, 1989) and many others.

The mechanism of action of 6-FQs, which involves inhibition of the intracellular enzymes DNA-gyrase and Topoisomerase IV, is well established (Weigel et al., 1998; Vila et al., 1997). However, as a first hypothesis, their broad spectrum of action seems to be due to its ability to cross bacterial envelopes and cytoplasmic membranes. Among others a "hydrophobic pathway" has been postulated. However, most of the studies devoid to elucidate its mechanism of entry evidenced the fact that 6-FQs main via of entry into the cytoplasm was through the porins (Berlanga et al., 2000a). On the other hand, the emergence of 6-FQs resistant strains is becoming an increasing problem, which jeop-

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PII: S0378-5173(02)00633-6

ardizes future application of this drug (Berlanga et al., 2000b). To be known, some of theses resistances occur by the activity and/or overexpression of protein efflux pumps. The activity of these proteins appears to depend on the ability of the surrounding inner monolayer of the cytoplasmic membrane to capture the drug (Van Bambeke et al., 2000). Once the drug is inserted at some level of the bilayer the molecule reaches by a still unknown mechanism the membrane protein and then it is expelled to the extracellular space. Whichever the case, influx through the lipid domains or efflux lipid mediated through membrane proteins; the interaction between 6-FQs and lipid membranes is a crucial point to understand their antimicrobial activities.

Ciprofloxacin (CPX), 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid is probably one of the most studied compounds and could be considered as the paradigm of the group. CPX is active against Gram-positive and Gram-negative bacteria and it is used either in human and veterinary diseases. Furthermore, CPX (and sparfloxacin) appear to be able to penetrate the acid–alcohol resistant bacteria. Thus, it became useful in the treatment against *Mycobacterium avium–M. intracellular* (Klopman et al., 1993) and *M. tuberculosis* occurred in secondary infections of AIDS patient's (Hooper and Wolfson, 1995).

Based on their physicochemical properties, 6-FQs appear as an ideal candidate for their encapsulation in liposomes (Majudmar et al., 1992). Unexpectedly, using passive methods, we found low encapsulation efficiencies (Montero et al., 1996), which evidenced some unusual properties of this drug. Notoriously, high trapping efficiencies were found using active methods (Maurer et al., 1998), which point, to the key role play by the amphipatic nature of the drug. Related with this, we revisited CPX microspeciation (Hernández-Borrell and Montero, 1997) and partitioning (Montero et al., 1997) and synthesized a homologous series of CPX in order to improve encapsulation efficiency in liposomes. We investigated the partitioning of their series in the *Eschericia coli* phospholipid extract-buffer model and found some relationship with antibacterial activities (Vázquez et al., 2001b). Directed to investigate phospholipid-CPX interaction, we exploited the fluorescence properties of CPX to localize the drug in bilayers by using quenching (Vázquez et al., 1998), anisotropy and binding experiments (Vázquez et al., 2001b,c; Merino et al., 2002). All these work, evidenced a low affinity of CPX for neutral and a preferential interaction with acidic phospholipids, which suggest the existence of an electrostatic interaction at surface level.

Several lines of evidences suggest that CPX could have, as other amphiphilic compounds, active surface properties. Thus, it appears that CPX might induce changes on neutral phospholipid (Montero et al., 1998; Grancelli et al., 2002) and can self-aggregate, at least at acidic pHs (Maurer et al., 1998). In this report, we further investigate the effect of CPX at the phospholipid interface. We will show results obtained applying anisotropy and quenching methods of fluorescence in combination with monolayers and epifluorescence microscopy. The aim of this paper is to complete previous works of our lab in order to gain insight into the relationship between the effects of CPX on phospholipid membranes and its antibiotic activity.

2. Materials and methods

2.1. Chemicals

L-α-1, 2-Dipalmitoyl-*sn*-glycerophosphocholine (DPPC, >99%), was purchased from Sigma, St. Louis, MO. Phospholipid purity was assessed by thin-layer chromatography. 1-Hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycerol-3 phosphocholine (Pyrene-PC) was obtained from Molecular Probes, Leiden, Holland. The fluorescent probe 1-palmitoyl-2-[12-{(7-nitro-2-1, 3-benzoxadiol-4-yl)amino}dodecanoyl]-*sn*-glycerol-3-phosphocholine (NBD-PC), was obtained from Avanti Polar Lipids. DPPC and NBD-PC were pure as judged by thin-layer-chromatography. Ciprofloxacin (CPX) was obtained from Cenavisa (Reus, S). Purity of the compound was assessed by IR and HPLC. The probes 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluene sulfonate (TMA-DPH) and (2-carboxyethyl)-1,6-diphenyl-1,3,5-hexatriene (PA-DPH were obtained from Molecular Probes (Eugene, OR). Quenchers were purchased from Sigma (St. Louis, MO). Buffer solution were HEPES, pH 7.40, 50 mM, $I = 0.15$ m, and acetate, pH 4.70, 0.15 M, $I = 0.15$ m. All other common chemicals were ACS grade.

2.2. Liposomes preparation

Chloroform/methanol (50:50, v/v) stock solutions of phospholipids and CPX were evaporated to dryness in a conical tube using a rotavapor. The resulting thin lipid film was then kept under high vacuum overnight to ensure the absence of organic solvent traces. Multilamellar vesicles were obtained by hydration in excess buffer. Thereafter suspensions were filtered through Nucleopore (Costar, Cambridge, MA) polycarbonate filters (200 nm nominal diameter) using an Extruder (Lipex Biomembranes Inc., BC) device. The size and polydispersity of liposomes were monitored by quasi-elastic light-scattering (QLS) using an Autosizer IIc photon correlation spectrophotometer (Malvern Instruments, UK).

2.3. Monolayer studies

Stock solutions of the lipid and CPX were made up in chloroform/methanol (3:1) to known concentrations of about 1 mM. DPPC and probe were mixed in chloroform/methanol (3:1) mixtures, typically a molar ratio (25:1), and spread at the air/water interface. The subphase was 5 mM HEPES buffer, pH 7.40, $I = 0.15$ m NaCl. Force-area isotherms of the DPPC:fluoroquinolone (1:1, mol:mol) mixed monolayers were obtained. The surface balance used in these studies has been described elsewhere (Nag et al., 1990). About 20 nmols of the probe and DPPC were spread from a Hamilton syringe and at least 10 min was allowed for solvent evaporation before compression and visual observation. The probe, NBD-PC was included at a concentration of 1 mol% in the lipid. The monolayers were formed over a buffer solution subphase and observed at ambient temperature (22 ± 2 °C). The monolayer was compressed at a barrier speed of 20 mm^2/s in 20 increments. Compression spread and the time allowed before compression were maintained constant in all experiments.

2.4. Fluorescence measurements

All measurements were carried out using an SLM-Aminco 8100 spectrofluorometer provided with a jacketed cuvette holder. The temperature was controlled within 0.1 °C using a circulating water bath (Haake, Germany). The excitation and emission slits were 4 and 4 nm and 8 and 8 nm, respectively.

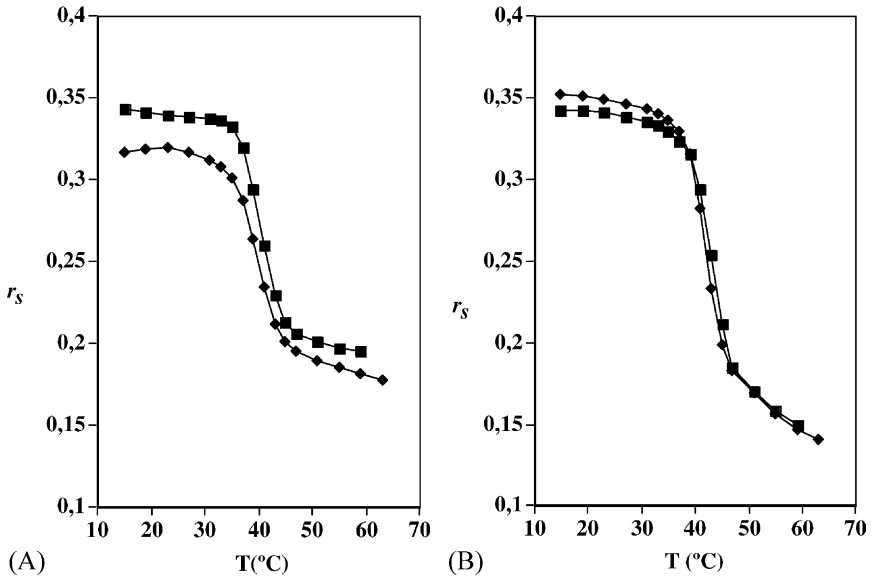

Fig. 1. Steady-state anisotropy of TMA-DPH (A) and PA-DPH (B) in absence (squares) and presence (diamonds) of 50 μM CPX for DPPC liposomes at pH 7.40 as a function of temperature. The total lipid concentration was 1 mM.

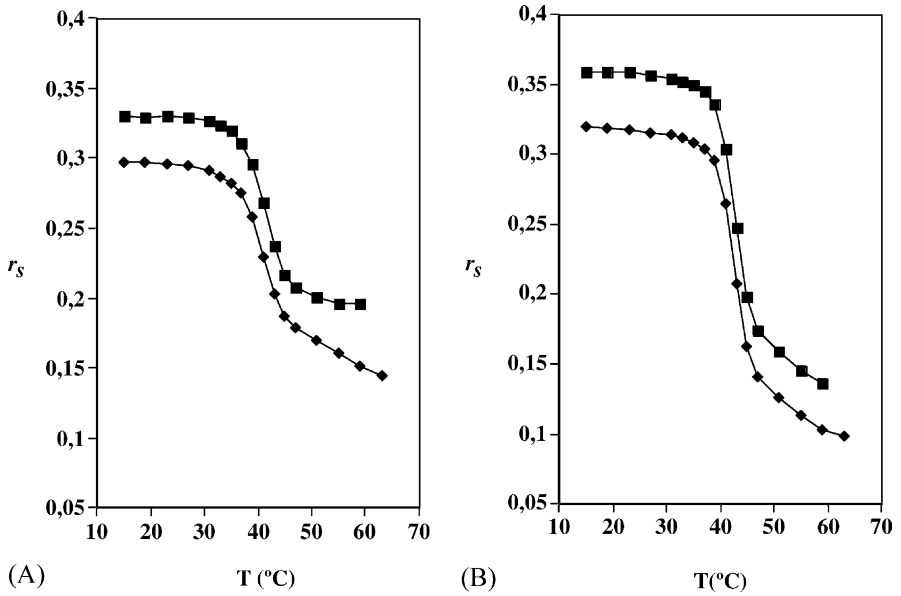

Fig. 2. Steady-state anisotropy of TMA-DPH (A) and PA-DPH (B) in absence (squares) and presence (diamonds) of 50 μM CPX for DPPC liposomes at pH 4.70 as a function of temperature. The total lipid concentration was 1 mM.

2.4.1. Fluorescence anisotropy experiments

The surface probes TMA-DPH or PA-DPH were incorporated in liposomes following incubation of 3 μl of concentrated stock solution (733 mM) of the probe in methanol in 1500 μl of liposome suspension for 30 min at 50 °C. The final lipid/fluorescent probe ratio was 500:1.57, mol/mol. The anisotropy was recorded in the range between 15 and 63 °C. Between 15–31 and 47–63 °C the anisotropy was recorded at four-degree intervals and between 31 and 47 °C at two-degree intervals. The excitation and fluorescence spectra of both probes were not independent of fluoroquinolone presence. Hence, excitation and emission wavelengths were 381 and 426 nm, respectively. The data obtained were analyzed according to the method previously described (Vázquez et al., 2001c). The polarization values reported in Figs. 1 and 2 are the average of three measurements.

2.4.2. Quenching experiments

We have followed the same methodology used in previous work (Vázquez et al., 1998). Briefly, liposomes encapsulating CPX (1 mM) or pyrene-PC were incubated in the presence of both quenchers iodobenzene and iododecanoic acid; which, were prepared before use in dimethylsulfoxide (2×10^{-2} M). Data were analysed according to the Stern–Volmer equation (Lakowicz, 1999).

3. Results and discussion

6-Fluoroquinolones are amphoteric molecules with two potential ionizable groups. Therefore, four different microspecies can be found in solution (neutral, zwitterion, positively and negatively charged) depending on the pH of the solution.[1] Hence, the kind of interaction force between CPX and the bilayer will depend on pH. It should be mentioned, that there was a controversy on the experimental determination of the microscopic constant (k_{21}) value and subsequent calculation of microspecies concentration. Indeed, k_{21}, is used as the starting point to calculate these concentrations. Values obtained for several 6-FQs by Takács-Novák et al. (1990) were objected by Ross and Riley (1992) with the argument that there was not a spectral shift associated with the protonation of the piperazinyl function.

[1] See scheme of microspeciation in Montero et al. (1997).

Table 1
The mole fraction of each microspecie for CPX at pH 7.4 were calculated using the macroconstant (K_i) and microconstants (k_{ij}) values previously published (Hernández-Borrell and Montero, 1997; Vázquez et al., 2001a,b,c)

pH	f [$HQ^{\pm}$]	f [HQ°]	f [H_2Q^{+}]	f [Q^{-}]
7.4	0.6605	0.2453	0.0545	0.0396
4.5	0.0543	0.0224	0.9232	0.0002

Within the context of 6-FQs-biomembrane interaction, the quantitation of the four microspecies become crucial for two main reasons: first, because only the uncharged microspecies can diffuse through the lipid bilayer, which is basic to predict the distribution of 6-FQs across the cytoplasmic membrane (Nikaido and Thanassi, 1993) and second, because only the charged microspecies will interact electrostatically with the bilayer (Vázquez et al., 2001b). Consequently, in order to calculate the concentration of each microspecies, we studied the applicability of the method originally developed for amino acids (Edsall and Wyman, 1958) and probed to be effective for CPX multiequilibria (Hernández-Borrell and Montero, 1997). Indeed, we found consistent values for microconstants and microspecies concentration (Table 1).[2] As can be seen there, while the uncharged and zwitterionic microspecies predominate at neutral pH, the positive microspecies predominates at acidic pH.

It has been elsewhere demonstrated (Maurer et al., 1998) that CPX at neutral pH show little tendency to bind neutral liposomes. Under similar conditions, however, a variation of approximately +6.52 mV of the electrostatic surface potential was observed (Vázquez et al., 2001b). Besides, there are strong evidences that CPX is located close to the liposome-buffer interface. Therefore, in order to investigate the contribution of positively and neutral microspecies to the neutral membrane insertion, steady-state anisotropy methods have been applied to study the localization of CPX in the bilayer.

[2] Results were dramatically affected by the accuracy of the experiments and on the instrumental sensitivity. This could explain the similarity between some of the K_1 and k_{21} values observed by Ross and Riley (1992). Importantly we found significant differences between both values for CPX (Hernández-Borrell and Montero, 1997 and Fig. 1 in Vázquez et al., 2001a).

Steady state anisotropy values (r_s) are high in the gel crystalline phase and low in the fluid phase. Thermotropic variations thereafter allow determination of the gel-to-fluid phase transition temperature (T_m) of the phospholipid bilayer. Fluorescence anisotropy experiments shown here were performed by using two probes TMA-DPH and PA-DPH, which are, aligned parallely to he phospholipid acyl chains by means of the DPH moiety. The charged groups amino (TMA-DPH) and propionic acid (PA-DPH) are both close to the bilayer surface and are suitable to explore such a region. It is believed that PA-DPH, however, is located more deeply in the bilayer than TMA-DPH (Kaiser and London, 1998).

The temperature dependence of TMA-DPH and PA-DPH fluorescence anisotropy for DPPC liposomes, in presence and absence of CPX, are shown in Fig. 1, at neutral pH, and Fig. 2, at acidic pH, respectively. The presence of CPX in DPPC liposomes caused small decreases of the TMA-DPH r_s values at neutral pHs (Fig. 1A). Similar behaviour was observed for TMA-DPH and PA-DPH r_s values at acidic pH (Fig. 2). However, at neutral pH, PA-DPH anisotropy values were almost non-affected by the presence of the drug and only marginal increases are observed below T_m (Fig. 1B). The values of T_m, calculated from the slope of the data fitted to sigmoid curve (data not shown), at neutral and acidic pH, showed small variations that fell within the experimental error. Moreover, at pH 4.70, where the positive microspecies predominates (see Table 1), the variations of T_m are even negligible. Therefore, we conclude that the main driving force involved in the interaction between CPX and DPPC liposomes should be necessarily hydrophobic rather than electrostatic. This is in agreement with a previous work carried out with surface charged liposomes and suggest that it is the electrostatic surface charge of the membrane which determines drug binding (Vázquez et al., 2001c; Merino et al., 2002). It is quite possible that this "capture" mechanism (Putman et al., 2000) (probably electrostatic) could precede CPX diffusion through naturally charged bilayers (see model of Furet et al., 1992).

In previous works (Vázquez et al., 1998) we have shown that CPX fluorescence in presence of DPPC liposomes, was moderately quenched by iodide and almost not quenched by acrylamide. There, we con-

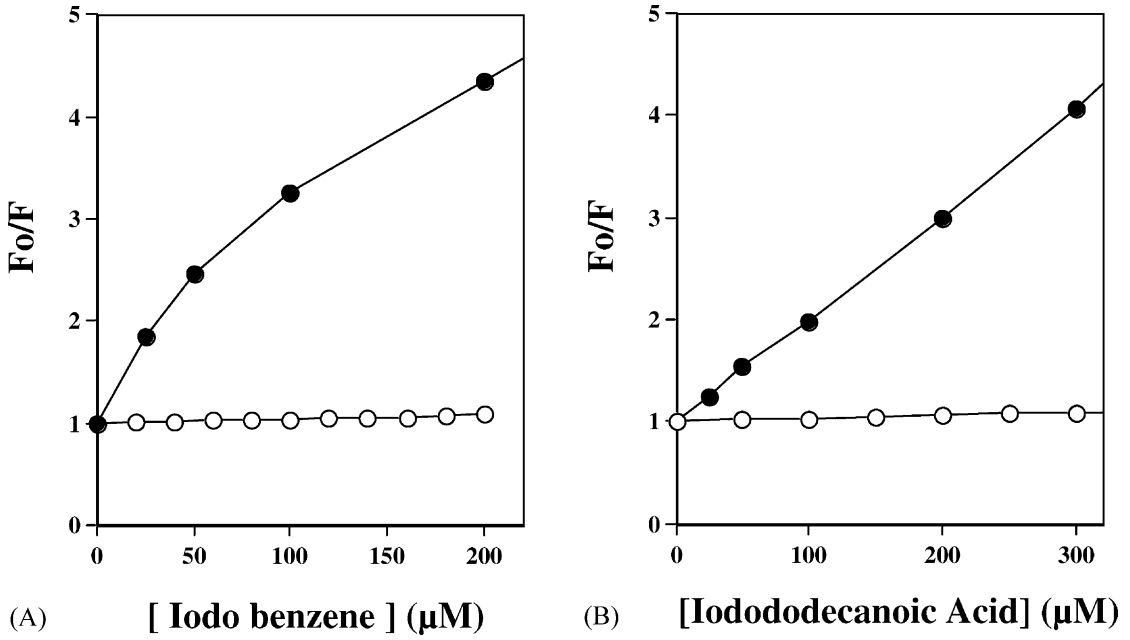

Fig. 3. Stern–Volmer plots of iodobenzene (A), and iododecanoic acid (B), for CPX–DPPC liposomes (open circles) and pyrene-PC DPPC liposomes (filled circles). The total lipid concentration was 1 mM.

clude that CPX should interact with the headgroup of the phospholipids. This is in agreement with the fluorescence anisotropy value presented above. Hence, to exclude the presence of CPX in the bilayer core, two different hydrophobic quenchers were used. As can be seen in Fig. 3A and B, neither iodobenzene nor iododecanoic acid (Deumié et al., 1995) were able to quench the fluorescence of CPX in presence of DPPC liposomes. By comparison, pyrene-PC fluorescence, (with the fluorophore in the core), was efficiently quenched by both iodide compounds. Therefore, CPX should be localized deeper in the membrane but not in the core, probably, in the vicinity of the first carbons of the acyl chains. This would explain the general decreases observed in the anisotropy values shown by the surface probes (Figs. 1 and 2).

In order to investigate the interfacial effects of CPX on DPPC molecules, we carried out a monolayer study complemented with observation of the interface with an epifluorescence microscope (Nag et al., 1990). Previously, we have demonstrated that CPX does not form monolayers at the air–water interface by itself (Montero et al., 1998). Here, Fig. 4 shows surface pressure-area isotherms for DPPC and

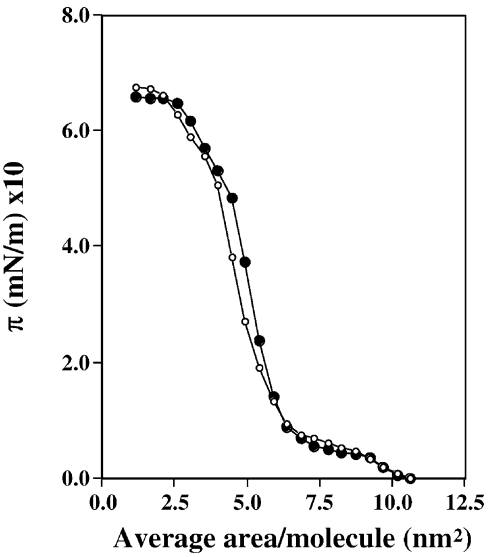

Fig. 4. Surface pressure vs. area per molecule of DPPC (filled circles) and DPPC/CPX (1:1) (open circles) isotherms.

DPPC/CPX (mol:mol) monolayers, both with 1 mol% of NBD-PC. The isotherm of DPPC monolayers showed familiar features than others previously published (Montero et al., 1998). Fig. 5A shows the typical images obtained by epifluorescence microscopy of DPPC monolayer at different pressures. Basically, for an easy interpretation, the fluorescent probe dissolves preferentially in the liquid expanded (LE) phase (light

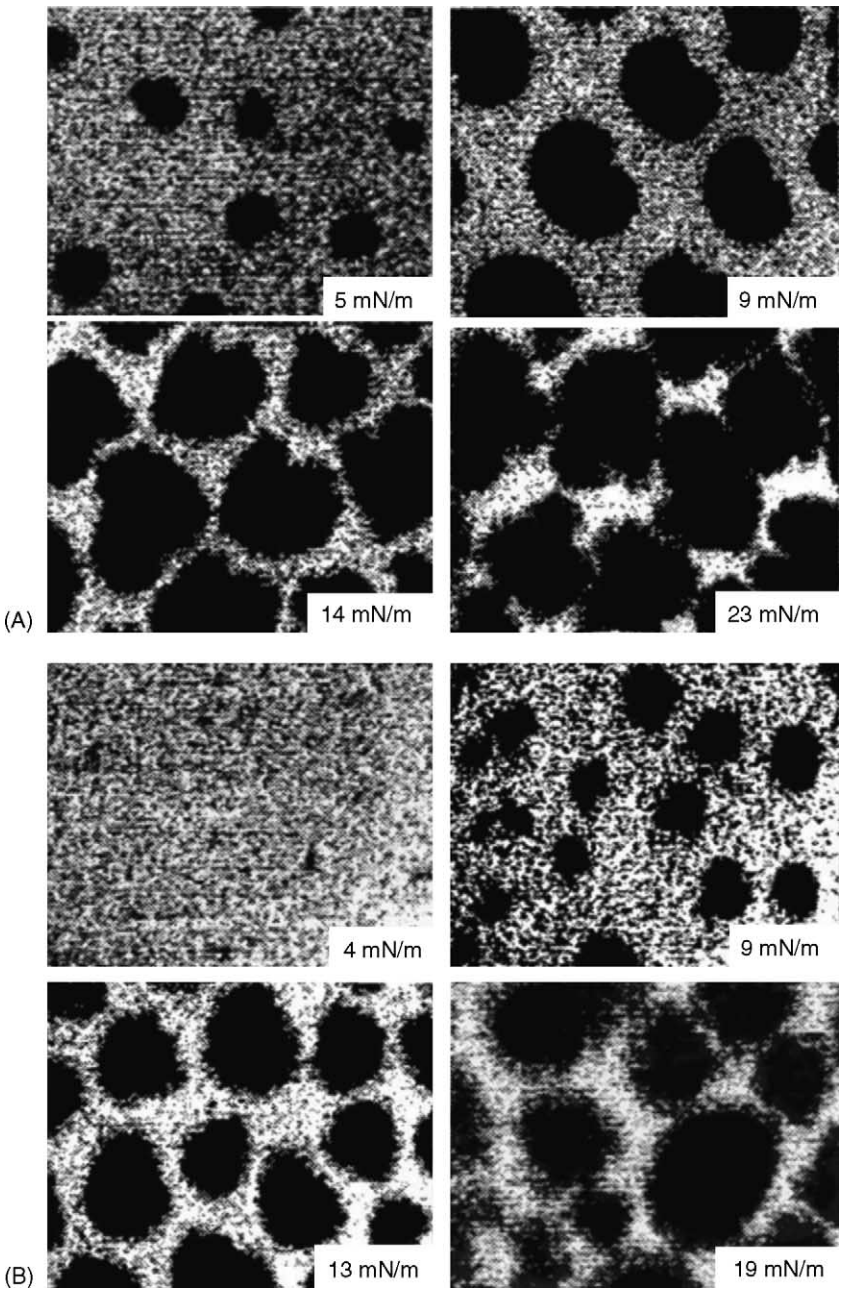

Fig. 5. Epifluorescence microscopy images obtained at different surface pressures from monolayers of DPPC (A), and DPPC/CPX (1:1) (B). Each monolayer contained 1 mol% NBD-PC. The dark probe excluding regions correspond to liquid-condensed domain in the pure DPPC.

areas). Conversely, the liquid condensed (LC) phases are the dark areas in the LE background. Thus, as compression progresses, LE–LC equilibrium displaces to the more condensed phase, which explains the increase in size and number of dark areas or domains. Of particular interest is the kidney-bean-shape (Nag et al., 1990) LC domains in a LE background found for DPPC films around 10 mN m^{-1} (Fig. 5A).

There were no significant differences in DPPC isotherm features up to the addition of 30 mol% CPX (data not shown). In Fig. 4 small shift of the isotherm toward lower area per molecule can be seen in presence of 50 mol% CPX. This reflects a non-ideal mixing behaviour between the drug and DPPC. Presumably, CPX is segregated or "squeezed-out" from the lipid environment. This behaviour has been encountered before with other 6-FQs (Nag et al., 1996) and explains the low affinity of CPX for neutral surfaces. When visualizing this monolayer (Fig. 5B), the existence of single LE phase was revealed at the lowest pressure (4 mN/m). At higher pressures the presence of CPX, however, induces changes in shape and reduction in size of LC domains. Actually, this is in agreement with the decreases in anisotropy values reported above (Figs. 1 and 2). Possibly this could reflect a reduction in the cooperativity of the LE–LC transition due to CPX presence.

4. Conclusions

The interaction of CPX with neutral membranes depends upon great number of physicochemical parameters. It is assumed that CPX can penetrate the cytoplasmic membrane by simple diffusion and most probably by partitioning and following an intermembrane concentration gradient. The results shown here, suggest that CPX is able to intercalate in a single monolayer leaflet. This phenomenon occurs irrespectively to the relative microspecies concentration. Consequently we concluded that at neutral pH and on neutral membranes the driving force for 6-FQs-biomembrane interactions is under these conditions hydrophobic.

Surface adsorption–insertion on inner/outer phospholipid monolayer of the cytoplasmic bacterial membrane is a first step before reaching the protein efflux pump complex. Recent findings of AFM on surface planar bilayers suggest that CPX is able to adsorb on the phospholipid surface (paper in preparation). Moreover, some experiments using black lipid bilayers suggest that CPX would be able to form pores.

Acknowledgements

This work has been supported by DGICYT (Grant PM98-0189) and Generalitat de Catalunya (Grant 2000SGR00017). We are indebted to Dr. Kevin W. Keough from The Memorial University of Newfoundland for facilities provided in the access to epifluorescence instrument. We are grateful to Dr. Manuel Prieto from the Centro de Química Física Molecular of Lisboa for useful insights into the analysis of the quenching and polarization data.

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