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Interaction of two phenothiazine derivatives with phospholipid monolayers

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Abstract

This paper addresses the cooperative interaction of two phenothiazine drugs, viz. trifluoperazine (TFP) and chlorpromazine (CPZ), with phospholipid monolayers as the model membrane system. Surface pressure and surface potential isotherms were obtained for mixed Langmuir monolayers of either dipalmitoyl-phosphatidyl-choline (DPPC) or dipalmitoyl-phosphatidyl-glycerol (DPPG) co-spread with TFP or CPZ. The changes in monolayer behavior caused by incorporation of a few molar ratio of drug molecules were practically within the experimental dispersion for the zwitterionic DPPC, and therefore a more refined analysis will be required to probe the interactions in an unequivocal way. For the charged DPPG, on the other hand, the surface pressure and the dipole moment were significantly affected even for TFP or CPZ concentrations as low as 0.002 molar ratio. Overall, the effects from CPZ and TFP are similar, but small differences exist which are probably due to the different protonation properties of the two drugs. For both drugs, changes are more prominent at the liftoff of the surface pressure, i.e. at the gas-condensed phase transition, with the surface pressure and surface potential isotherms becoming more expanded with the drug incorporation. With DPPG/CPZ monolayers, in particular, an additional phase transition appears at higher CPZ concentrations, which resembles the effects from increasing the subphase temperature for a pure DPPG monolayer. The dipole moment for DPPG/CPZ and DPPG/TFP monolayers decreases with the drug concentration, which means that the effects from the charged drugs are not associated with changes in the double-layer potential. Otherwise, the effective dipole moment should increase with the drug concentration. The changes caused in surface pressure and dipole moment by small concentrations of TFP or CPZ can only be explained by some cooperative effect through which the contribution from DPPG molecules changes considerably, i.e. even DPPG molecules that are not neighbor to a CPZ or TFP molecule are also affected. Such changes may occur either through a significant reorientation of the DPPG molecules or to a change in their hydration state. We discuss the cooperativity semi-quantitatively by estimating the number of lipid molecules affected by the drug interaction. CPZ and TFP also affect the morphology of DPPG monolayers, which was confirmed with Brewster angle microscopy. The biological implications from the cooperative, non-specific interaction of CPZ and TFP with membranes are also commented upon.

Keywords: Phenothiazines; Chlorpromazine (CPZ); Trifluoperazine (TFP); Zwitterionic; Anionic phospholipid monolayers; Dipalmitoyl-phosphatidyl-choline (DPPC); Dipalmitoyl-phosphatidyl-glycerol (DPPG); Surface potential; Brewster angle microscopy (BAM); Interaction model

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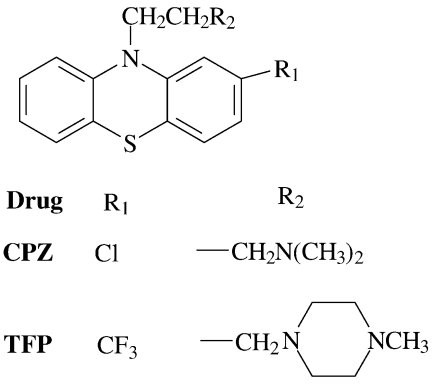


Fig. 1. Chemical structure of chlorpromazine (CPZ) and trifluoperazine (TFP).

1. Introduction

Biological membranes are constituted mainly by phospholipids, glycolipids and cholesterol, providing a framework to which proteins can be attached. The phospholipid molecules, in particular, self-assemble into bilayers, thus providing boundaries for cells and subcellular organelles and maintaining chemical gradients. The activity of many membrane-bound proteins, such as receptors, ion channels and enzymes, depend on the physical state or phospholipid composition of the membrane. Though the important role of the biological membrane is not disputed, many physicochemical properties associated with its function are still not understood. Yet, this is crucial not only for understanding physiological mechanisms but also for developing new applications, e.g. use of liposomes (self-closed structures of phospholipids) for encapsulating specific vectors to transport drug and target specific places to be treated [1]. Moreover, many pharmacologically active molecules have intracellular targets and elucidation of the mechanisms affecting the passive diffusion of compounds through the lipid bilayer is thus of primary importance.

Trifluoperazine (TFP) and chlorpromazine (CPZ), whose chemical structures are shown in Fig. 1, are phenothiazine derivatives classified as antipsychotic agents. Both drugs are commonly recommended in psychiatric books where neuroleptic sedative treatment is needed. TFP is also a well-known calmodulin antagonist, and the structure of TFP-calmodulin complex has been resolved to the atomic level [2]. This effect is related directly to the interaction of this drug with a protein, a specific target. At concentrations above 100 μM, CPZ makes holes of 14 Å in diameter in red cell membranes [3], which is associated with changes in the membrane structure. At concentrations above 40 μM CPZ renders the cells permeable to low-molecular weight substances. Both CPZ and TFP have amphiphilic properties and self-aggregate at a critical concentration, forming micelle-like structures [4], which could be certainly relevant for their interaction with the membrane and fluidizing effect on its structure. Molnár et al. investigated the reversal of multidrug resistance (mdr) in tumor cells, and of the resistance of *Escherichia coli* to ampicillin and other antibiotics with phenothiazines. They concluded that the resistance effect is reversed by phenothiazines due to the changes caused in the membrane [5]. This also motivated the research on the interaction of phenothiazines with model membranes using different techniques such as ^{13}C and ^{31}P nuclear magnetic resonance and calorimetry. Frenzel et al. [6] have combined NMR and calorimetry to study the CPZ interaction with DMPC, from which they infer that the drug penetrates into the hydrophobic core, increasing the mobility of the hydrocarbon chains. Hendrich et al. [7] studied the TFP interaction with DPPC liposomes using calorimetry and fluorescence spectroscopy techniques and found that TFP induces domain formation in DPPC model membranes.

The use of Langmuir monolayers of phospholipids as a membrane model has regained interest recently [8–12]. Phospholipid monolayers provide a highly informative approach for studying drug-lipid interactions, as there is no curvature effect (unlike vesicles) and the lateral packing can be precisely controlled [15]. Moreover, the control of the state of the hydrocarbon chains and the relative concentration of the drug (both on the monolayer and in the subphase) allow a molecular control of the parameters associated with the drug effects. Jutila et al. [9] investigated the effect of CPZ and other phenothiazine derivatives in the lateral het-

erogeneity of DPPC/brainPS and eggPC monolayers. In this case, the drugs induced an increase in the average area per molecule. Fluorescence microscopy revealed that the size and shape of the domains in the coexistence region are affected by the presence of the drugs [9]. Agasøsler et al. [10,11] observed a large effect on the surface area (expansion) of acidic glycerophospholipids spread on a subphase containing micromolar concentrations of CPZ. A systematic study on this expansion [11] revealed two different behaviors for the expansion depending on the bulk concentration of the drug and the phospholipid used on the monolayer. The interaction of dipyridamole (DIP) with DPPC monolayers has been studied in our laboratory [8], where surprisingly large impact was found for low DIP:DPPC molar ratios (one DIP molecule for 500 DPPC molecules). Such a low DIP concentration indicates that the association of the drug to the monolayer itself cannot explain the macroscopic changes observed in monolayer properties. It has been postulated that the drug induces some cooperative effect on the phospholipid monolayer [8,12].

The present study was undertaken to further explore the interaction between TFP and CPZ with glycerophospholipids using monolayers at the air–water interface (Langmuir monolayers). We selected two phospholipids with the same acyl chains and different head groups: 1,2-dipalmitoyl-sn-3-glycero-[phospho-rac-(1-glycerol)] (DPPG, a charged anionic phospholipid) and 1,2-dipalmitoyl-sn-3-glycero-phosphatidylcholine (DPPC, a neutral zwitterionic phospholipid). Using these phospholipids allows evaluation of the importance of the charge and hydrophilic character on the interaction of the drugs with model membranes. A number of studies were carried out with phosphatidylserine monolayers instead of DPPG [9–11], but the head group of phosphatidylserines is more complex, combining a positive charge between two separated negative charges. Surface pressure isotherms provided information about changes on the mean area per lipid molecule, while surface potential isotherms provided information on the reorientation of the mean dipole moment per molecule. The combined information from these techniques allowed us to observe considerable effects upon monolayer properties already at very low relative drug concentrations.

2. Materials and methods

1,2-dipalmitoyl-sn-3-glycero-[phospho-rac-(1-glycerol)] (DPPG, purity > 99%) was purchased from Avanti Polar Lipids, Inc. 1,2-dipalmitoyl-sn-3-glycero-phosphatidylcholine (DPPC, purity > 99%), and the drugs, TFP and CPZ, were purchased from Sigma-Aldrich Co. All the chemicals were used as received without further purification, and the solvents (chloroform and methanol) were of HPLC grade. Experiments were carried out using a thermostated bath at 22.0 ± 0.5 °C (except for the temperature dependence study of the pure DPPG monolayer). Surface pressure and surface potential measurements have been carried out on a KSV 5000 Langmuir through in a class 10 000 clean room. The surface pressure π was determined using the Wilhelmy plate method, where the plate was made from chromatography paper – Whatman Chr1. The surface potential ΔV was measured using the vibrating plate method ($\nu = 300$ Hz) with a KSV Kelvin probe with both reference and vibrating plate electrodes made from platinum and the probe located at approximately 2–3 mm above the water surface. The system was controlled by a computer allowing to obtain simultaneously the surface-pressure isotherm ($\pi - A$ isotherm) and the surface-potential isotherm ($\Delta V - A$ isotherm) on a single run. After spreading, the solvent was allowed to evaporate for 8 min. Film compression using two symmetrically moving barriers was carried out at a constant barrier speed (10 mm/min for DPPC monolayers and 8 mm/min for the DPPG monolayers, corresponding to ~ 0.02 Å^2/molecule/s or ~ 0.03 Å^2/molecule/s). No differences on the isotherms were found using compression speeds between 15 and 5 mm/min.

Brewster angle microscope (BAM 2 plus, NFT) is equipped with a red laser with emission wavelength of 690 nm and a power of 30 mW. All the system is operated through a specially adapted program developed by NFT. To obtain the correct top view of the images the program performs a special scanning, focusing on different regions of

the image and composing a whole focused image. Then the same program performs the geometrical correction to obtain the top view. All the images were carried out with a 10x objective allowing a resolution of 2 μ. The microscope is mounted on a NIMA Langmuir through and the same compression speed used for recording the surface potential and surface pressure isotherms was used during the procedure to obtain the images. The compression speed is an important parameter to control in taking the images, as high compression rates produce fingered domains, similar to the observations made by Vollhardt et al. [14].

To study the interaction of biomolecules (drugs, proteins, etc.) with surface phospholipid monolayers, usually the phospholipid monolayer is compressed on a subphase where the biomolecule has been dissolved [10,11]. Another approach consists of injecting a solution with the biomolecule in the subphase and allowing for the diffusion of the molecule in the subphase towards the monolayer. Other authors compress the phospholipid monolayer and monitor the changes after spreading the solution with the biomolecule directly over the phospholipid monolayer [13]. In all of these approaches there is no control of the exact number of biomolecules that interact with the phospholipid monolayer. The later method, i.e. spreading the biomolecule solution over the pre-formed monolayer, has the additional disadvantage of possible loss of phospholipid molecules to the subphase. When the biomolecules are dissolved in the subphase, the effects on the monolayer are interpreted using Scatchard plots [11], where the knowledge of a binding constant is necessary. In order to avoid these difficulties we have used the co-spreading procedure [8] in which both the lipid and the (partially hydrophobic) drug molecules are dissolved in a common, volatile solvent, thus forming the spreading solution. This procedure exhibits the following advantages: (1) if the solubility of the drug in aqueous solvents is sufficiently low or the affinity of the molecule to the phospholipid is high enough, the number of drug molecules interacting with the lipid monolayer is precisely known; (2) in contrast to the injection approach, the lateral distribution of the drug at the interface is homogeneous and time-independent.

DPPG monolayers were prepared from a chloroform/methanol (9:1, v:v) stock solution of 0.7 g/l (or $\sim 9\times10^{-4}$ M). DPPC monolayers were prepared from a pure chloroform solution of 0.5 g/l (or $\sim 7\times10^{-4}$ M). For the mixed monolayers including drugs other stock solutions were prepared with similar molar concentrations. This concentrated solution was diluted 10, 100 and 1000 times the concentration of the phospholipid stock solution (i.e. 9×10^{-5} M, 9×10^{-6} M, and 9×10^{-7} M, respectively). These 3 solutions of drugs were used for mixing in the corresponding volume relations to have the desired relative drug/phospholipid concentrations. The mixing process was performed in a separate third tube just by shaking the solution, after which an aliquot was spread on the water surface taking care to keep constant the number of phospholipid molecules in the monolayer. Finally, each monolayer was compressed once, and to establish error bars for some concentrations the experiments were repeated using different stock solutions. Ultra pure water produced by a Nanopore water purification system coupled with a Milli-Q water purification system (resistivity = 18.2 MΩ cm) was used as subphase.

In terms of drug concentrations, the procedures adopted here may be compared to those employed by Agasøsler et al. [10,11] and Beurer and Galla [16], in which CPZ was dissolved in the subphase for the study of its interaction with phospholipid monolayers. They found that for concentrations lower than 1×10^{-6} M any expansion produced by the interaction of the monolayer with the drug in the subphase is lost in the errors. In our case the maximum concentration studied was 20 mol% of TFP ([TFP]/[DPPG] = 0.2). If all this TFP were dissolved in the subphase (if there were no interactions and all TFP molecules were lost to the subphase, which we know does not occur as will be shown later), the final concentration in the subphase would be $\sim 6\times10^{-9}$ M of TFP. This value is three orders of magnitude lower than the minimum concentration required by Beurer and Agasøsler for detecting some expansion on the phospholipid monolayer. The stability of the mixed monolayers was tested at some concentrations (0.01 drug/lipid molar ratio) in our case by cycling (compressing and decompressing) the

same monolayer several times, with an interval of 30 min. between compression and decompression. No differences were observed in the surface pressure or surface potential, which means that there is no loss of molecules.

3. Results and discussion

The effect from CPZ and TFP on the zwitterionic DPPC monolayers, observed in surface pressure and surface potential isotherms, is relatively small for low drug concentrations, as can be seen in Figs. 2 and 3. In these figures, the area is given in terms of area per DPPC molecule and therefore any change from pure DPPC can be attributed to the interaction with the drug molecules. For TFP, in particular, a slight expansion (Fig. 2a) and increase in surface potential (Fig. 3A) are observed in the LE–LC phase transition, while the changes in the isotherms are practically negligible at high pressures, denoting that the drug is expelled from the interface. For both drugs, the effect on the limiting area of the monolayers (indicated by arrow d) in Fig. 2A,B is slight, the main effect occurring in the plateau region, producing an increase in pressure and shortening of the plateau, especially at the end of the coexistence region (indicated by arrow c) in Fig. 2A,B. Also, the liftoff of the surface pressure (indicated by arrow a) in Fig. 2A,B is shifted to higher areas per lipid, indicating a slight monolayer expansion. Note that the surface potential isotherms display features that may be correlated with the surface pressure isotherms. For DPPC, changes in slope marked by arrows in Fig. 3 appear at approximately 95 $Å^2$, which corresponds to the liftoff of surface pressure, i.e. to the gaseous → liquid expanded phase transition, and at ~76 $Å^2$, corresponding to the beginning of the liquid expanded ↔ liquid condensed coexistence region, i.e. the plateau in the surface pressure isotherm. For CPZ, the changes are practically within the dispersion of the experimental data, and no conclusion can be drawn from its effects on the DPPC monolayers. For both CPZ and TFP, therefore, further analysis will require more sensitive measurements or techniques to probe the interaction at low concentrations.

For the charged DPPG monolayers, the change in slope in the surface potential isotherm corresponds to the liftoff in surface pressure or the gaseous → liquid condensed phase transition. The effects from both TFP and CPZ are readily apparent in the surface pressure and surface potential isotherms of Figs. 4 and 5, especially in the phase transition. The liftoff of the surface pressure (indicated by arrows in Fig. 4a,b) is shifted significantly to higher areas with increasing concentrations of TFP and CPZ. At higher CPZ concentrations (Fig. 4b), the π—A isotherm changes in shape that resembles the plateau of the pure DPPC system. This characteristic feature does not appear for the highest TFP concentration studied, which points to a difference in the associating properties with the DPPG monolayer for these drugs. The limiting areas of the DPPG monolayers are not strongly affected by CPZ in the concentration range studied; however, for TFP a considerable expansion is produced with a relative drug concentration of 0.05 M ratio. In Table 1 shows the areas at the liftoff for the mixed drug/DPPG monolayers.

Fig. 6 shows that for both CPZ and TFP, the liftoff area for the surface pressure increases abruptly at very low drug concentration, and then less steeply for higher concentrations. We shall discuss this result later on. At fixed surface pressures, the area increases linearly with the concentration for TFP, with higher slopes at lower surface pressures. This means that TFP appears not to be entirely excluded from the film interface, even at high surface pressures. For CPZ, on the other hand, the effects from the drug are much smaller at high surface pressures, probably because CPZ is excluded from the film interface. It should be mentioned that attempts to produce monolayers from pure CPZ or TFP solutions were unsuccessful, for these drugs dissolve well in the water subphase.

The linear increase in area per DPPG molecule with TFP concentration suggests that the mixed monolayer may be thought of as consisting of free lipids—with area per molecule identical to that of a pure DPPG monolayer, A_{Lfree} – and lipids interacting with the drug (complexed lipids), which supposedly will occupy a different area per mole-

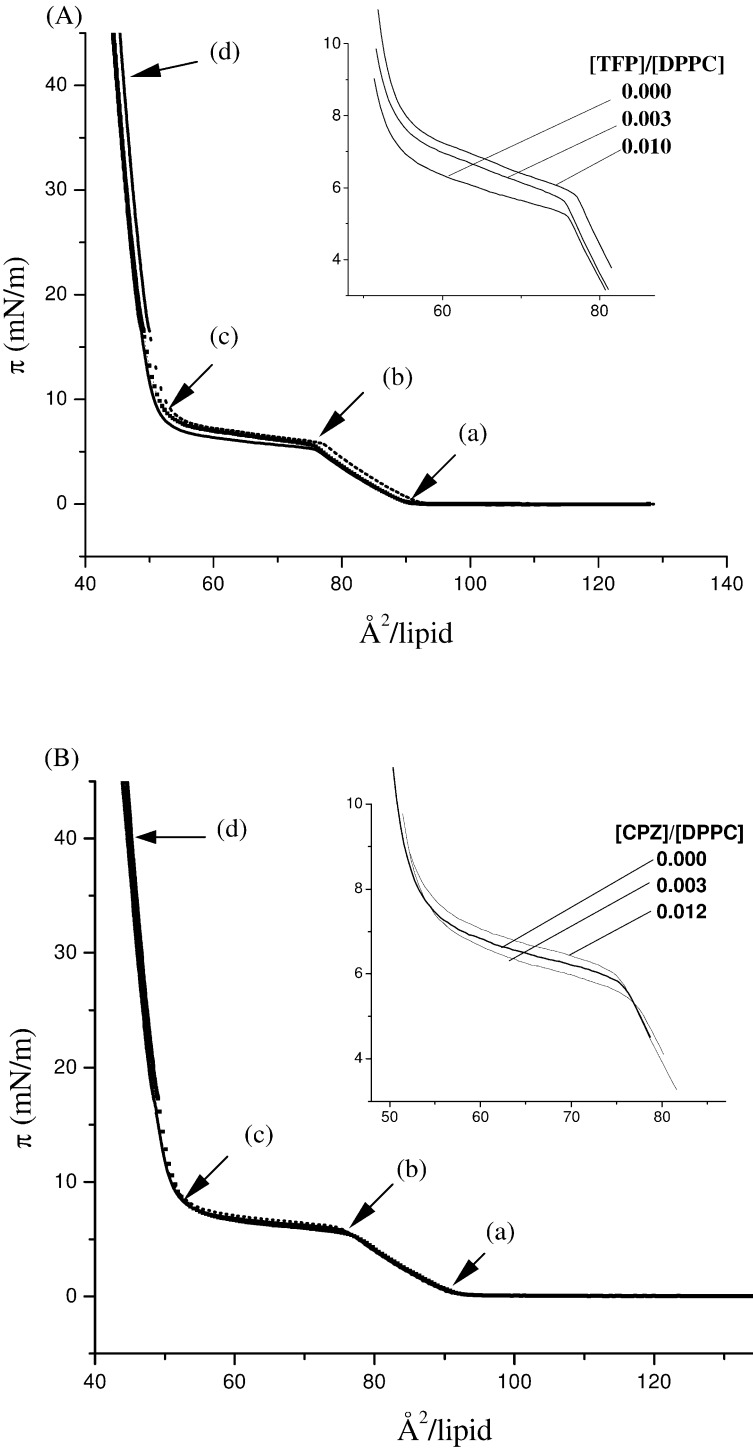
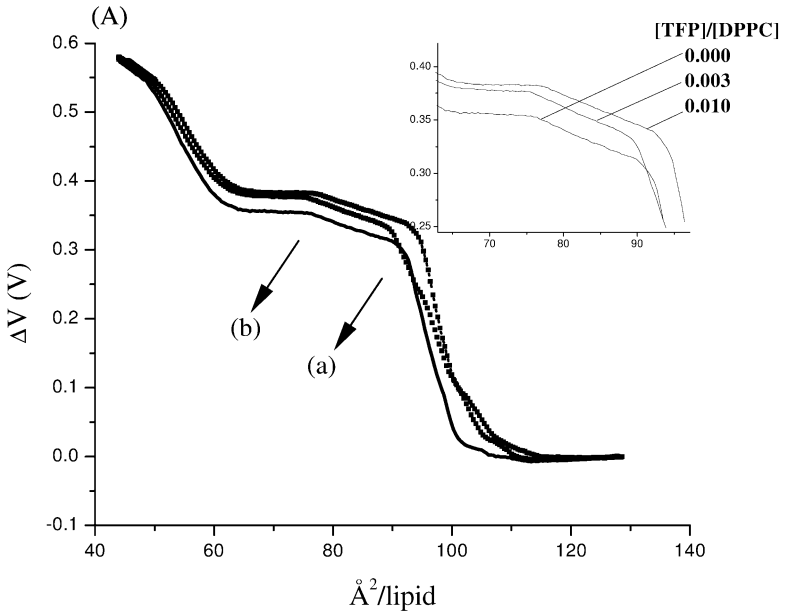


Fig. 2. (A) Surface pressure isotherms for the mixed TFP/DPPC monolayers. (B) surface pressure for the CPZ/DPPC monolayers. Not all the concentrations are plotted to avoid overcrowding the figures. The points indicated with arrows correspond to (a) liftoff, (b) beginning of the plateau, (c) end of the plateau and (d) extrapolated area from 40 mN/m.



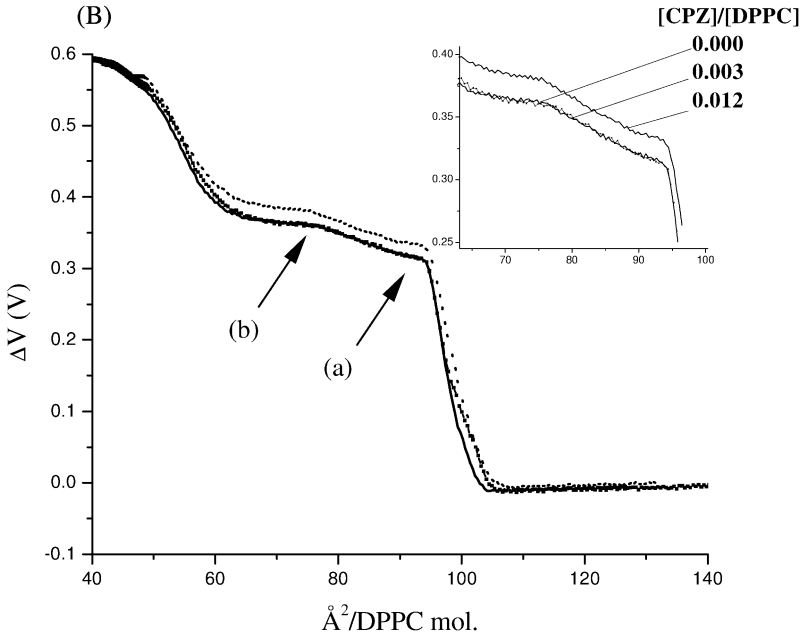


Fig. 3. (A) Surface potential of the mixed monolayers TFP/DPPC. (B) surface potential for the mixed monolayers CPZ/DPPC. Arrows indicate the corresponding points in the surface pressure isotherm: (a) liftoff and (b) beginning of plateau.

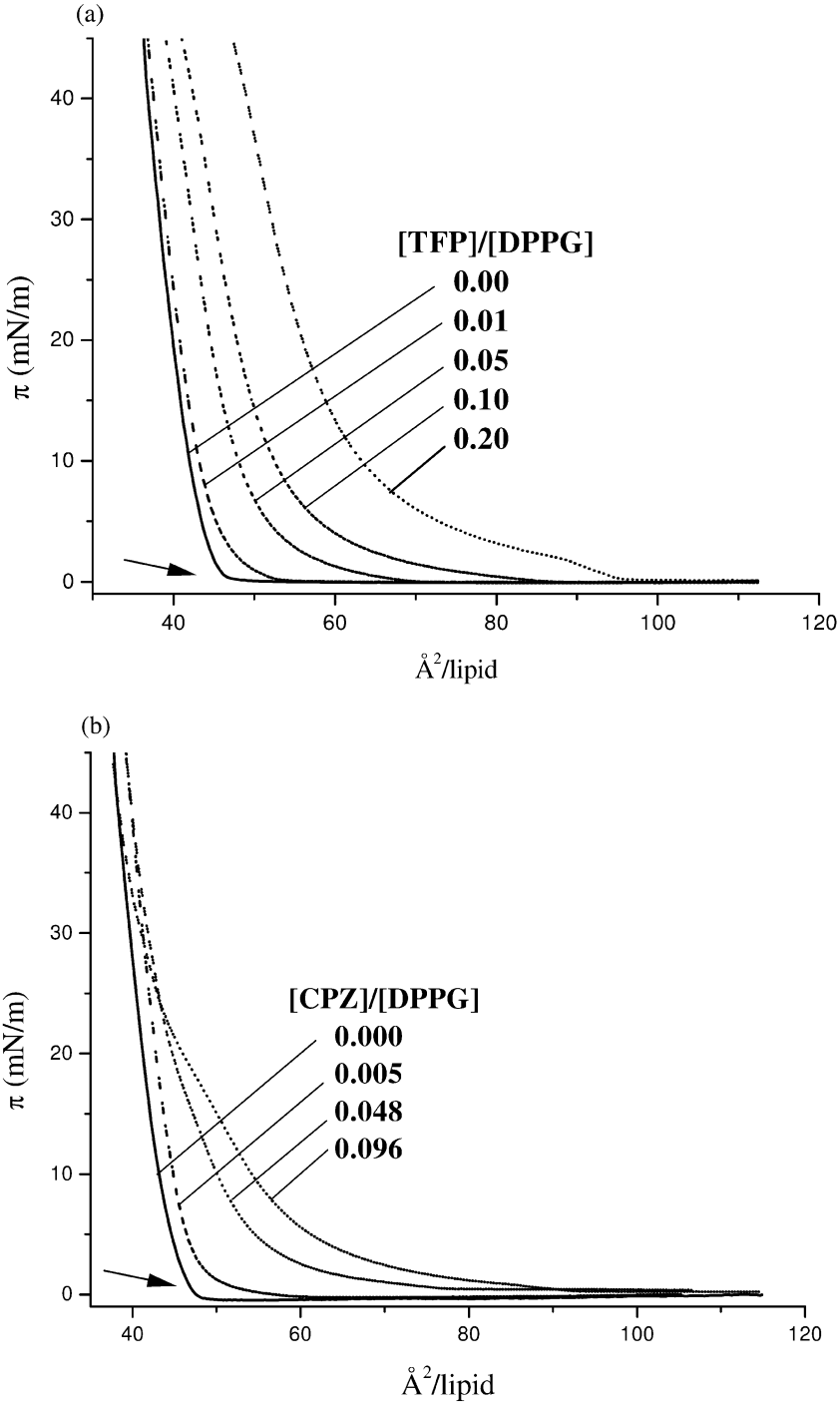


Fig. 4. (a) Surface pressure isotherms for the mixed TFP/DPPG monolayers. (b) surface pressure for the CPZ/DPPG monolayers. Not all concentrations are plotted to avoid overcrowding the figures. The arrows indicate the liftoff of the pressure.

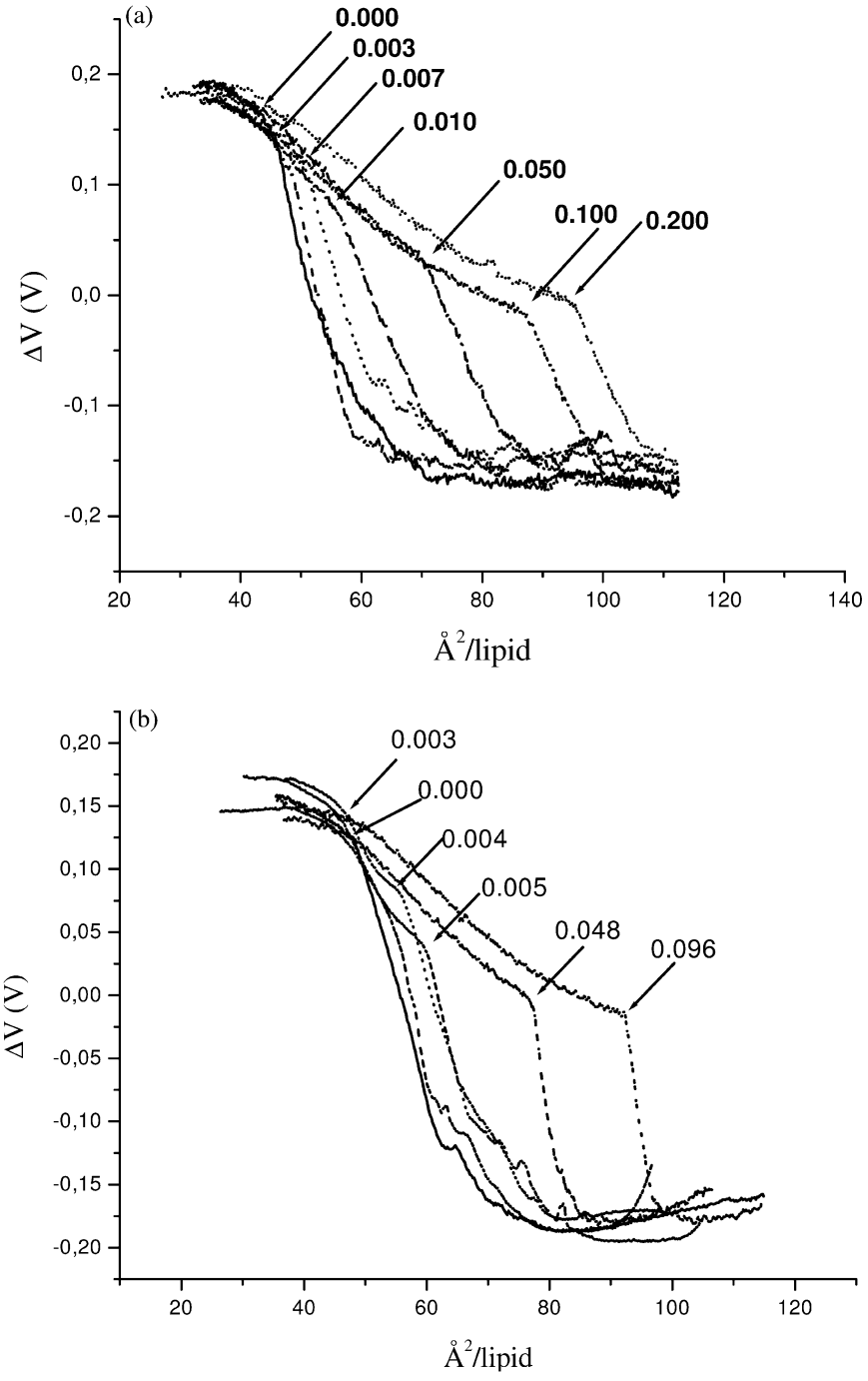


Fig. 5. (a) Surface potential of the mixed monolayers TFP/DPPG. (b) surface potential of the mixed monolayers CPZ/DPPG. The arrows indicate the kink in the surface potential related to the gaseous→liquid condensed phase transition and the liftoff of the surface pressure.

Table 1
Area at the liftoff for the mixed drug/DPPG monolayers

Relative molar concentration	Liftoff	
	CPZ/DPPG monolayers (± 1 Å^2)	TFP/DPPG monolayers (± 1 Å^2)
0.000	47	46
0.001	51	46
0.002	55	49
0.003	56	48
0.004	57	51
0.005	60	52
0.007	–	51
0.01	–	57
0.05	78	71
0.1	92	87
0.2	–	96

cule, A_C, this area being the mean area in the complex. In fact, even if there were no interaction, A_C would still be different from A_{Lfree} because of the incorporation of the drug molecule. The area per lipid molecule in the mixed monolayer is then

$$A = \chi n A_C + (1 - n\chi) A_{\mathrm{Lfree}} \quad (1)$$

where χ is the ratio between the number of drugs and lipid molecules (i.e. concentration)[1] and n is the number of lipid molecules affected by the drug. Eq. (1) may be rearranged in the form

$$A - A_{\mathrm{Lfree}} = \chi n (A_C - A_{\mathrm{Lfree}}) \quad (2)$$

which confirms the expectation that the change in area should be proportional to the drug concentration. By fitting the data of Fig. 6a,b with Eq. (2), we obtain the area values $n(A_C - A_{\mathrm{Lfree}})$ shown in Table 2, which are particularly large especially for the liftoff of the surface pressure. During the fitting sessions we confirmed that the linear coefficient in Eq. (2) is indeed zero. The linear correlation parameter was always close to one, demonstrating the good quality of the fittings (see the continuous lines in Fig. 6). Note from Eq. (2) that if the area difference $A - A_{\mathrm{Lfree}}$ goes to zero $A_C - A_{\mathrm{Lfree}}$ also tends to zero, indicating that the drug is expelled from the monolayer or is associated just peripherically, exactly what we found from the fittings at high pressures for the CPZ (see Table 2 and the surface pressure isotherms in Fig. 4b). At the liftoff, the resulting areas are 25 and 50 times the area of pure DPPG (see Table 2). Such large values can only be explained if the lipid molecules interacting directly with the drug have their area changed substantially, in other words A_C would be very different from A_{Lfree}, which is unrealistic, or if the number of lipid molecules affected by the drug, n, is very large. We have no way to determine n precisely as it depends on how each individual lipid molecule will have its area changed, but rough estimates can be made. For example, for a purely electrostatic interaction we should expect n to be 1 for CPZ (protonated) and 2 for TFP (doubly protonated). If other neighboring molecules are affected, n would increase to 5 or 6, as suggested for CPZ interdigitizing in liposomes of mixed phospholipids [17]. In any case, such values of n are not sufficient to lead to the large area changes in Table 2, and therefore the inevitable conclusion is that cooperativity exists so that the number of lipid molecules affected is large. That is to say, not only the neighboring lipid molecules are affected. Large values for n have indeed been proposed to explain cooperativity in multilamellar suspensions [18], where n is of the order of 600.

We now focus on the surface potential results or the effective normal component of the dipole

[1] The possible aggregation of the drug in the monolayer was completely disregarded due to the very low concentrations used both as stock solutions and in the spreading solution.

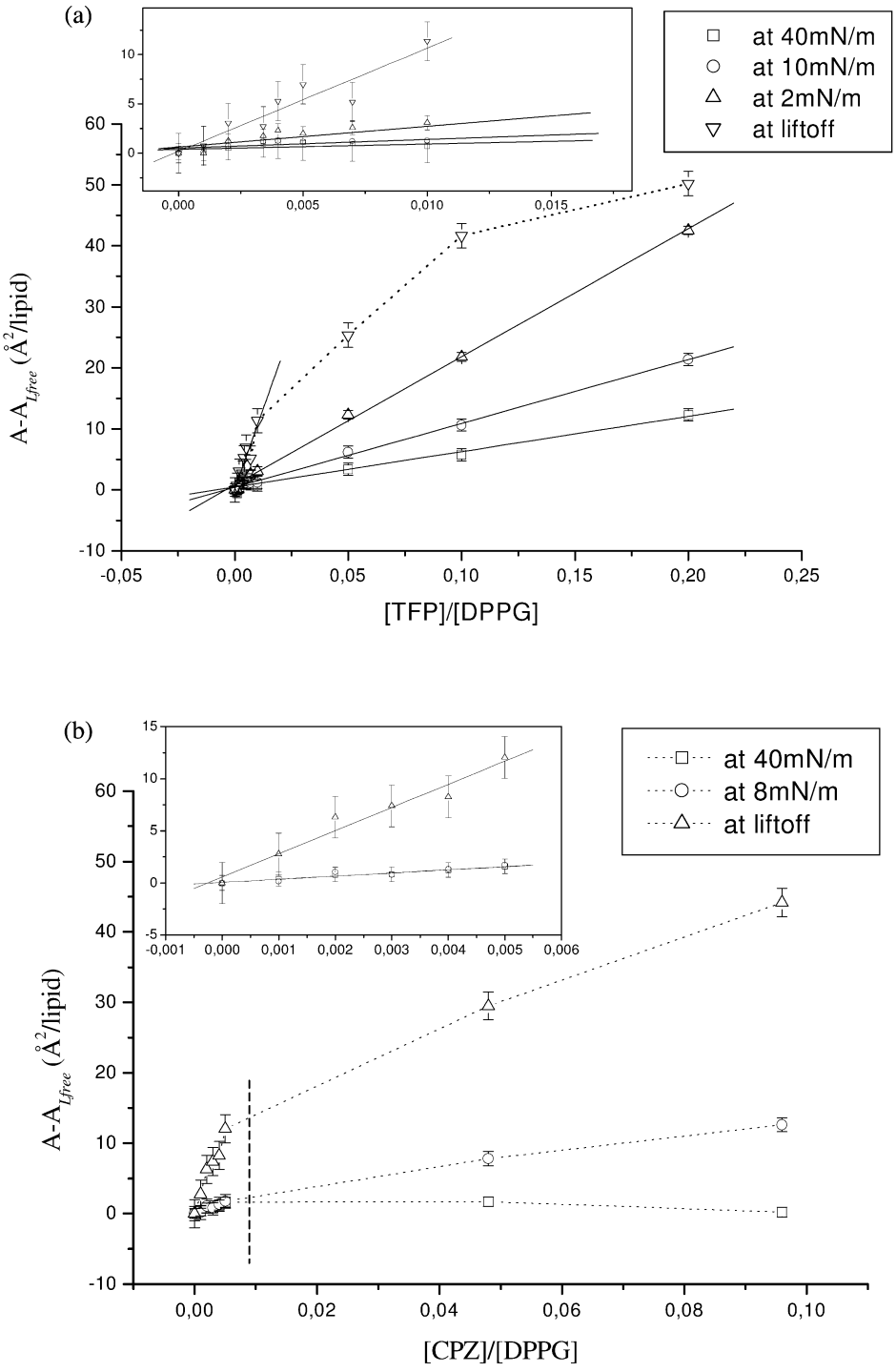


Fig. 6. Plot of area difference between mixed and pure DPPG monolayers at the phase transition and at constant pressures vs. concentration of the drug. (a) TFP/DPPG and (b) CPZ/DPPG. The solid lines represent the fittings according to Eq. (2).

Table 2
Results from fitting the data in Fig. 6a,b with Eq. (2)

π(mN/m)	$n(A_C - A_{Lfree})$ (Å^2/lipid)	
	TFP/DPPG monolayers	CPZ/DPPG monolayers
40	57±2	303±20
30	66±2	432±90
15	88±2	329±30
10	105±2	335±50
8	116±2	342±60
4	159±2	–
2	210±3	–
Liftoff	1048±156	2222±200

moment per molecule, $\mu_\perp$, which may be obtained from the measured surface potential with the Helmholtz model in which the molecules are considered as an array of dipoles:

$$\mu_\perp = \Delta V\, A \varepsilon_0 \tag{3}$$

where ε_0 is the vacuum permittivity and the monolayer was assumed to have an effective dielectric constant of 1 (a discussion on the value of dielectric constants for Langmuir monolayers is found in Ref. [19]). Using the normal component of the dipole moment instead of comparing directly the surface potential is a better choice when evaluating the expansion of the monolayer such as observed in our experiments. In principle, changes in the conformation of the lipid molecule, reorganization of the hydrating water and also the drug molecules can alter $\mu_\perp$. Note also that this effective $\mu_\perp$ includes the contribution from the double-layer that is formed between the charged DPPG monolayer and the water subphase. It is this negative contribution that causes the surface potential for DPPG monolayers to be negative at large areas per molecule. Fig. 7 shows that large changes in $\mu_\perp$ occur at the phase transition (liftoff area) for both CPZ and TFP, whereas for the latter reasonable effects remain at low surface pressures, up to 15 mN/m. For CPZ/DPPG monolayers there is no significant change in dipole moment at non-zero pressures.

That DPPG monolayers are at least partially charged on a pure water subphase is well known, and confirmed by the negative surface potential at large areas per molecule in Fig. 5. The question then arises of whether CPZ and TFP are charged at the interface. In aqueous solutions CPZ has a pK_a of 9.3 while TFP has two pK_a values, 8.0 and 4.0 [20]. At the DPPG interface, pH$_s$ is lower than in the bulk, according to the Henderson–Hasselbalch equation

$$\mathrm{pH_s} = \mathrm{pH_b} + \frac{e\Psi_0}{2.3\ kT} \tag{4}$$

where pH$_b$ is the bulk pH, e is the elementary charge, Ψ_0 is the double-layer potential, k is the Boltzman constant and T is the absolute temperature. Taking from our experimental results, $\Psi_0 = -0.16 \pm 0.02$ V, pH$_b$=5.8 and the temperature used, a pH$_s$ of 3.2 is obtained for the interface, which indicates that both TFP and CPZ are completely protonated (charged).

The incorporation of such positively charged drugs on a negatively charged, DPPG monolayer could affect $\mu_\perp$ in the following ways: (i) by reducing the effective surface charge of the DPPG monolayer, which decreases the negative contribution from the double-layer, thus increasing $\mu_\perp$ (ii) by providing a normal component to the dipole moment from the drug itself. (iii) by altering the dipole contribution from DPPG owing to reorientations in the lipid caused by the binding of drug. It is obvious that for the very small concentrations of CPZ and TFP, contributions (i) and (ii) are negligible. Furthermore, the overall effect from the drugs has been to decrease $\mu_\perp$ (Fig. 7), contrary to what is expected from the changes in the double-layer potential (item ii) above). It is concluded,

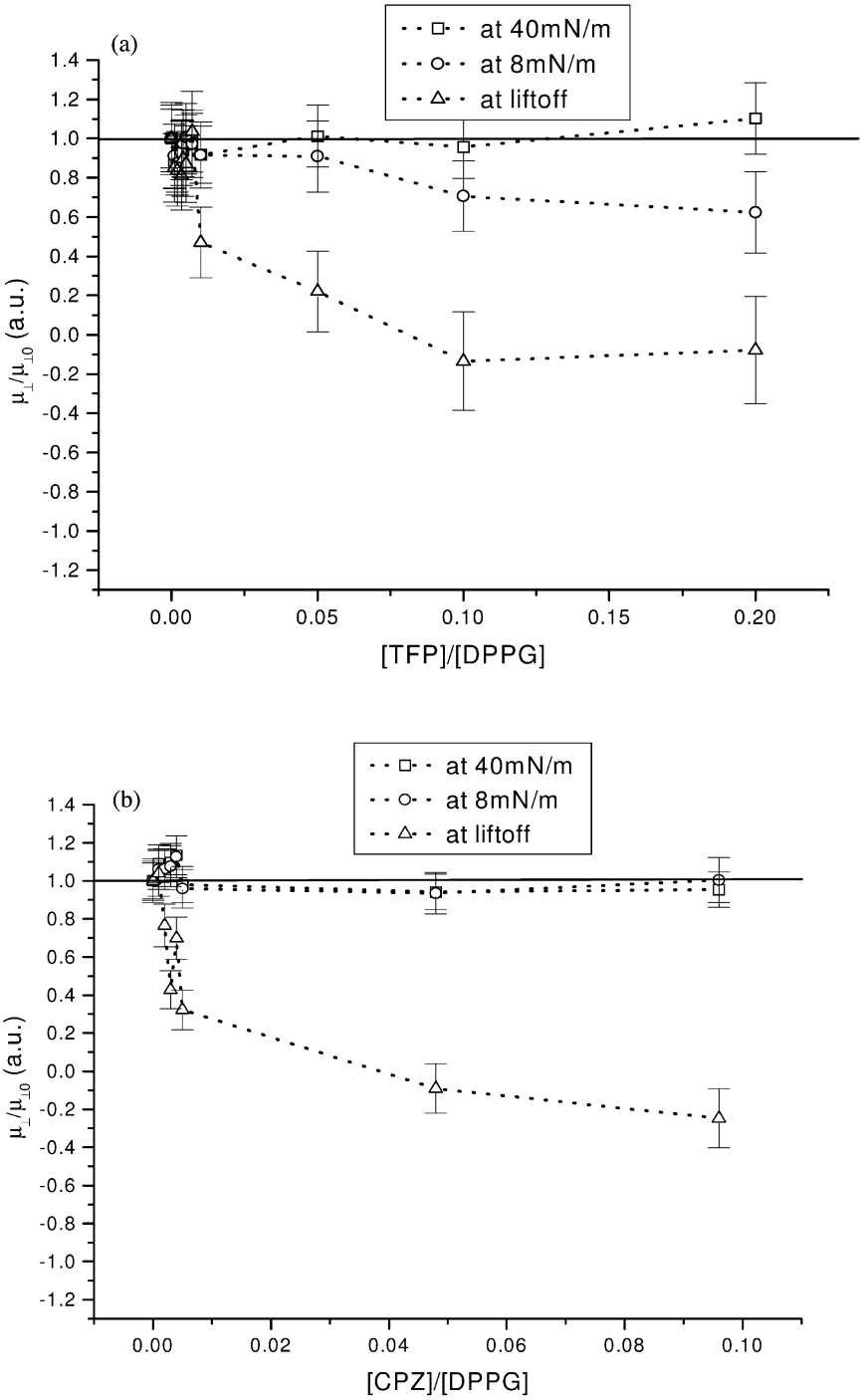


Fig. 7. Normalized changes in the dipole moment for the mixed monolayers: (a) TFP/DPPG monolayers and (b) CPZ/DPPG monolayers.

therefore, that the most important effect from the incorporation of CPZ and TFP is in altering the vertical component of the dipole moment of DPPG. For very low concentrations, i.e. lower than 0.01 M ratio (or 1 mol%), where sizable effects are observed, this can only occur if the DPPG molecules are affected cooperatively. That is to say, similarly to the conclusions drawn from the surface pressure-area isotherms, the drugs affect the lipid packing in such a way that DPPG molecules that are not neighbor to the drug molecules are also affected. This is indeed consistent with our previous findings with DPPC/dipyridamole interactions [8], and appears to be a general feature of phospholipid monolayers. The biological implications of this conclusion will be discussed later on. For the higher concentrations of TFP and CPZ (0.2 M ratio), one should expect the double-layer contribution to decrease significantly. However, this does not occur experimentally, which probably means that this change in double-layer potential is compensated by changes in the vertical component of the dipole moment of DPPG molecules.

Having observed that the major effects from CPZ and TFP on DPPG monolayers occur at the phase transition, we wondered whether such findings could be related to temperature effects. Here, we follow the approach proposed by Beurer and Galla [16], who suggested that the monolayer properties of DPPC containing CPZ are comparable to those from pure DPPC monolayers at higher temperatures. Following this idea, Agassosler [11] also compared the CPZ/phosphatidylserine monolayers with the pure monolayer at higher temperatures. To check this point, we investigated pure DPPG monolayers at different temperatures, and the changes in area and dipole moment, in relation to the monolayer at 22 °C, are depicted in Fig. 8. A plateau-like feature appears at temperatures above 26 °C, with the pressure at the plateau increasing with the temperature and with no significant monolayer expansion at high pressures. The liftoff area increases with temperature up to 26 °C, after which it remains constant. At 26 °C the dipole moment is reversed at the liftoff, and then it remains constant for higher temperatures (Fig. 8). As the temperature is increased, there is some effect on the dipole moment for higher pressures. For pressures above 8 mN/m, changes in dipole moment are within the experimental dispersion of the data. Even though the detailed features in the surface pressure and dipole moment data may differ for the drugs and the temperature dependence depicted in Fig. 8, a comparison indicates that the drug effects may be correlated with the temperature effects, at least for the phase transition region. For DPPG/CPZ monolayers, the plateau-like feature in the surface pressures at higher CPZ concentrations resembles the change occurring at approximately 26 °C for pure DPPG monolayers, though it must be admitted that such a feature is not present in DPPG/TFP isotherms. Furthermore, the dipole moment at the liftoff area decreases considerably with both CPZ and TFP concentration, similarly to what is observed as the temperature is increased in Fig. 8. In summary, CPZ and TFP appear to have a similar cooperative (see below) effect to the 'melting' of hydrocarbon tails, which has been suggested to explain the temperature dependence of phospholipid monolayers [11,16].

The findings mentioned above are confirmed on the domain morphology studies using Brewster angle microscopy. For DPPC, the change in morphology upon introducing TFP or CPZ is very small, consistent with the findings for surface pressure and surface potential isotherms discussed in Figs. 2 and 3. For DPPG, on the other hand, the interaction with CPZ or TFP affected film morphology as follows: (i) at large areas per molecule (starting at $\sim$80 Å^2/lipid), 'collar'-like structures are formed on the CPZ or TFP/DPPG mixed monolayers, which are absent in the pure DPPG monolayer, as can be seen in Fig. 9 and in the images by Vollhard et al. [14] taken at $T=26$ °C. These 'collar'-like structures are observed for both drugs for molar ratios between 0.002 and 0.01. For the lower concentration, 0.002 M ratio, these structures coexist with completely dark regions. For CPZ or TFP concentrations up to 0.01 M ratio, upon compression the morphology resembles that of a pure DPPG monolayer, though the stages at which particular features are observed differ. The complete evolution of the morphology of the domains is illustrated in Fig. 10 for several

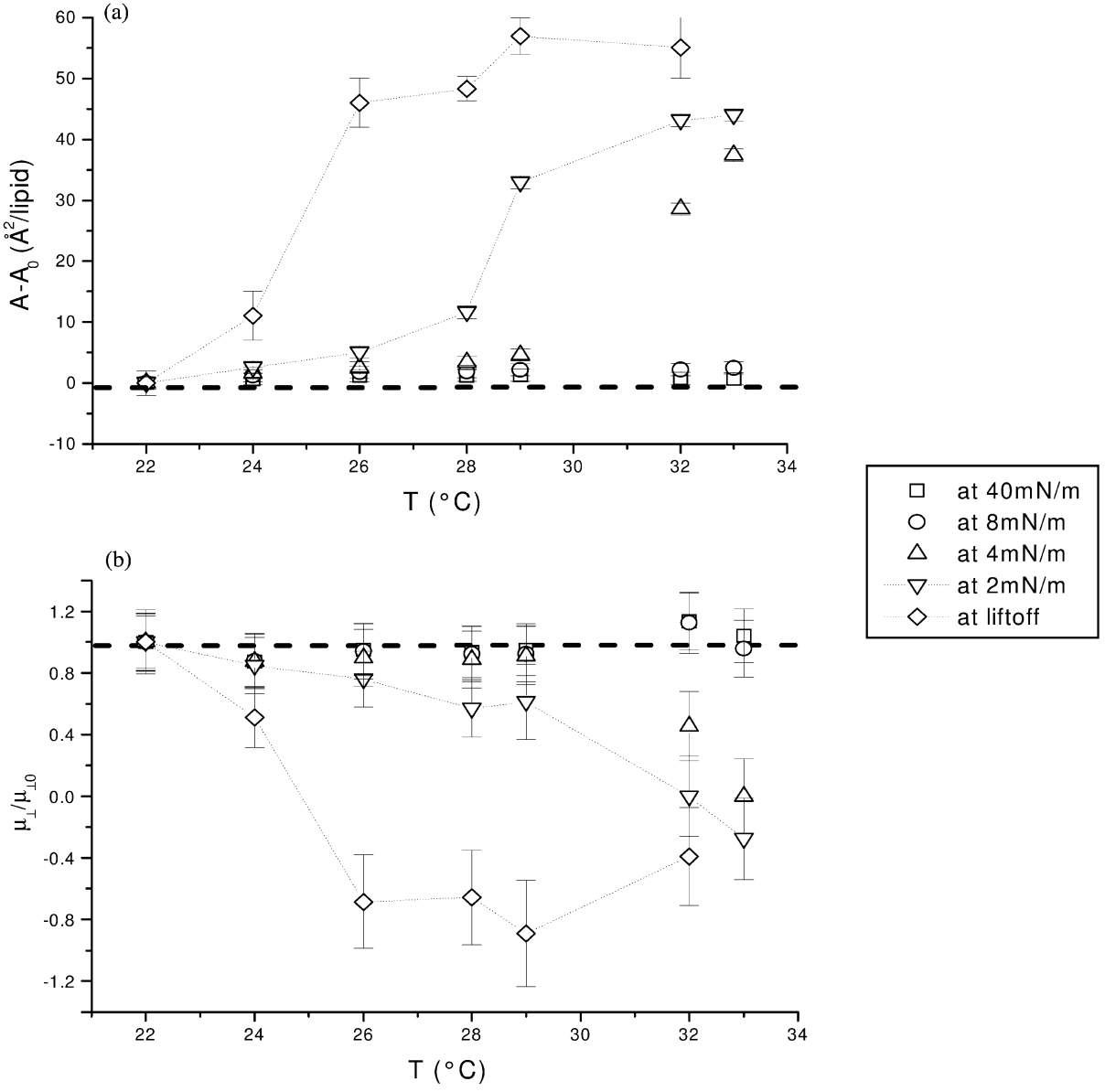


Fig. 8. (a) Temperature dependence of the expansion (b) Temperature dependence of the dipole moment per molecule.

values of areas per lipid. The most important feature is that as the monolayer approaches the liftoff point, the morphology tends to that of Fig. 9. Similar 'collar'-like structures were also observed in fluorescence microscopy experiments in the same range of phenothiazine concentrations (Caetano, unpublished results) (ii) At high phenothiazine concentrations, however, drastic changes are noted as shown in Fig. 11 for 0.1 M ratio of CPZ. The characteristic feature of the monolayer at these concentrations is the fluidity, with similar circular domains observed in Fig. 10 (image b) for 0.005 M ratio of phenothiazine, but the 'collar'-like structures are not observed. Another feature

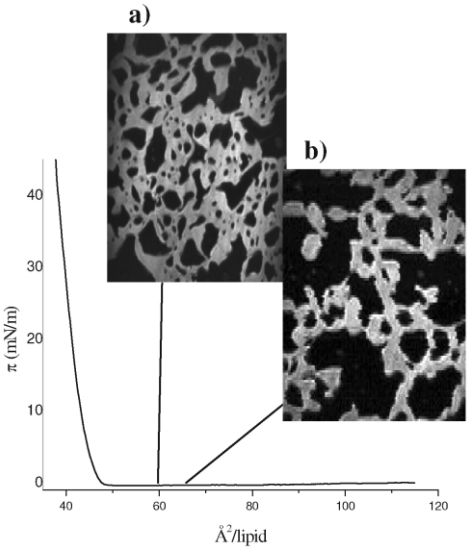


Fig. 9. BAM images with the corresponding pressure isotherm of pure DPPG monolayer. Image (a) taken at 60 Å²/lipid and (b) taken at 65 Å²/lipid.

of monolayers with CPZ is a change in domain shape during compression, illustrated in Fig. 11 (image a), which does not appear for pure DPPG (see Fig. 9) or for the mixed monolayer with TFP (the surface pressure isotherms are also different at this concentration, see Fig. 4). For the mixed monolayers with TFP, the circular domains are observed at all stages of compression. These circular domains observed for high drug concentrations are similar to those observed in pure DPPG by Vollhard et al. [14] at 26 °C, and have similar dimensions to the structures that compose the 'collar'-like structures, with a diameter ranging from 7 to 16 μm. The fluidizing effect of the drugs at high concentrations is also observed as an increase in the number for circular domains observed both in BAM images as well as in fluorescence microscopy (Caetano, unpublished observations).

Finally, the results presented here from surface potential and surface pressure isotherms and BAM point to the existence of a cooperative effect through which DPPG molecules have their properties changed even when they are not neighbor to a drug (CPZ or TFP) molecule. This is precisely the conclusion drawn from the studies of dipyridamole interaction with DPPC monolayers [8,12]. It is also consistent with the findings by Frenzel et al. and Hendrich et al. [6,7], who showed with calorimetry that for concentrations as low as 2 mol% and 1 mol% of CPZ (or 0.02 to 0.01 M ratio), respectively, the pretransition in liposomes of DPPC is completely suppressed. This indicates that the effects of the drug on the membrane model starts for concentrations lower than 1 mol%, though the latter authors [6,7] did not stress this point. The changes in monolayer properties in a cooperative way may occur not only due to reorientation and different packing of the phospholipid molecules, but also in the hydration layer in the vicinity of the headgroups. For instance, Agasøsler [10,11] attributed the effect of CPZ on phosphatidylserine monolayers to a cooperative effect due to a hydrogen bond network, inducing a reorientation of the lipid. To distinguish between effects from the hydration layer, Brewster angle microscopy experiments (operating for reflectivity to determine changes on the layer thickness) combined with the techniques presented in the present work are in progress.

The cooperativity and the shift in temperature of the main phase transition of phosphocholine bilayers has been used to classify the interactions with small molecules [18], particularly with regard to the location of these guest molecules. The calorimetric results of Hendrich et al. [7] on the interaction of DPPG with TFP indicate that this drug is partially buried in the hydrocarbon core of the bilayer, interacting primarily with the C2–C8 methylene region of the hydrocarbon chains, close to the carbonyl group. Such interaction decreases the temperature of the main transition and increases the width of the transition peak, with the enthalpy of transition being practically unaffected. Nerdal et al. [17] combined NMR and calorimetry experiments on mixed liposomes of DPPC/PS and CPZ to propose a molecular model in which the positive charge of CPZ is close to the phosphate group of the lipid while the heterocyclic rings are located at the hydrophobic–hydrophilic interface. Our data for CPZ on monolayers are consistent with the latter model and also with the lowering in temperature of the phase transition on liposomes

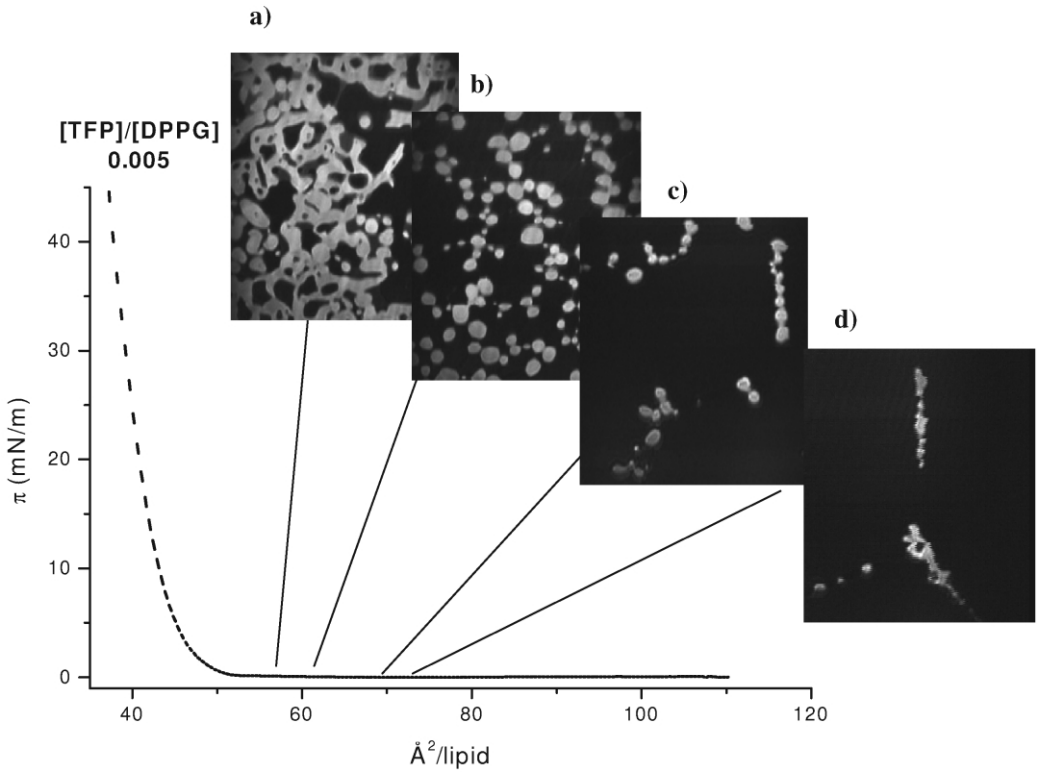


Fig. 10. Typical BAM images for selected stages of the compression isotherm for a mixed TFP/DPPG monolayer (0.005 M ratio). Image (a) taken at 57 Å^2/lipid, (b) at 61 Å^2/lipid, (c) at 71 Å^2/lipid and (d) at 73 Å^2/lipid.

[17,18]. It can be recalled from Fig. 4b that the behavior of DPPG/CPZ monolayers resembles that of pure DPPG monolayers above 26 °C.

With regard to the biological implications, the cooperativity effect involving the membrane demonstrated in this work may help elucidate mechanisms for physiological action of drugs, in particular such drugs with relatively non-specific effects upon the membrane. It is known that the high efficiency of drug action – thus requiring a very small concentration of the drug in living bodies, 10^{-7} to 10^{-9} M – may be explained by the mediation of membrane receptors (e.g. proteins) [21]. The interaction with specific receptors may be also the reason why drugs—such as dipyridamole and TFP or CPZ—that display similar physicochemical properties and interact similarly with model membranes, have such distinct physiological roles. Dipyridamole is a vasodilator [22] while TFP and CPZ are antipsychotic drugs [23]. However, these 3 drugs share a common physiological action as antioxidizing agents, in a process that apparently does not depend on specific receptors, but is rather associated with membrane interactions [22,24]. Accordingly, the similar physiological action implies similar interactions with the membrane, as observed in Langmuir monolayers for dipyridamole [8] and CPZ and TFP discussed in the present work. These interactions are non-specific, as can be noted by the much lower association constants of these drugs with the membrane (10^4–10^5 mol^{-1}) [21] in comparison with 10^7–10^9 mol^{-1} for hormones [21]. Furthermore, in the absence of protein mediation, high pharmacological efficiency relies on cooperative effects with the membrane, similarly to the cooperativity in the phase transitions that do not require specific interactions, as postulated here.

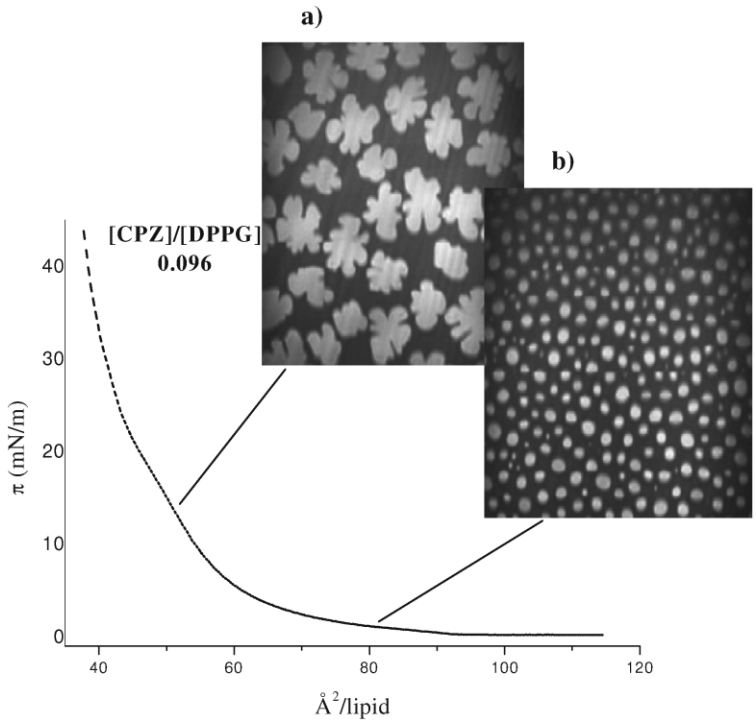


Fig. 11. Change in domain shape of a mixed DPPG monolayer (with 0.1 M ratio of CPZ) during continuous compression. Image (a) taken at 80 Å^2/lipid and (b) taken at

4. Conclusions

We have shown that TFP and CPZ affect the zwitterionic DPPC monolayers only to a small extent. For CPZ, in particular, the changes caused by interaction with the drug are practically within the dispersion of the experimental data. More refined analysis will be required to draw useful conclusions from these systems. With the charged DPPG, however, reasonably large effects are observed, especially at the phase transition. That the effects from TFP and CPZ are much larger in DPPG than in DPPC is in agreement with the work by Agasøsler [11], where interaction of CPZ with phosphatidylserines was investigated in detail. For DPPG/CPZ, much more expanded monolayers are obtained, with the appearance of a phase transition (plateau-like confirmed with the change in domain shape in Fig. 11) at higher concentrations. At high pressures, there is little increase in area, which means that the drug is being excluded from the interface. The surface potential isotherms are more expanded than for pure DPPG. At the liftoff for the pressure, which coincides with a change in behavior in the surface potential curve, there is significant change in the surface potential and dipole moment. The latter decreases with increasing CPZ concentration, and changes are very small at high pressures. The decrease in dipole moment with the CPZ concentration means that the double-layer is probably not affected by the presence of the drug. Otherwise, the dipole moment should increase rather than decrease, as CPZ is expected to be positively charged and would cancel part of the negative charge of the DPPG monolayer. The changes in surface potential occur already at very low CPZ concentrations, which could not affect directly the double-layer contribution. Moreover, for such small concentrations one cannot attribute the changes in dipole moment to the dipoles of the CPZ itself. The inevitable conclusion is that some cooperative

effect must be acting so that the contribution from DPPG molecules changes considerably, i.e. even DPPG molecules that are not neighbor to a CPZ molecule are also affected. Such changes in dipole moment may occur either through a significant reorientation of the DPPG molecules or to a change in their hydration state—which affects both the local dielectric constant and the orientation.

The DPPG/TFP monolayers are also more expanded than pure DPPG, even though there is no appearance of a further phase transition. However, unlike for CPZ, the increase in area per molecule remains at high pressures. This means that TFP molecules remain in the monolayer, which is consistent with the linear increase in the area per molecule with the TFP concentration. The behavior of the surface potential and dipole moment is very similar to that of CPZ, but with changes remaining at low surface pressures.

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