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Cooperativity of phospholipid reorganization upon interaction of dipyridamole with surface monolayers on water

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Abstract

Results from various surface sensitive characterization techniques suggest a model for the interaction of the piperidinopyrimidine dipyridamole (DIP) — known as a vasodilator and inhibitor of *P*-glycoprotein associated multidrug resistance of tumor cells — with phospholipid monolayers in which the drug is peripherally associated with the membrane, binding (up to) five phospholipids at a time. These multiple interactions are responsible for a very strong association of the drug with the lipid monolayer even at exceedingly low concentrations (~ 0.2 mol%). Electrostatic interactions and hydrogen bonding are likely involved in the binding of DIP to DPPC. Cooperative effects among the lipids are invoked to explain the macroscopically measurable changes of lipid monolayer properties even when only one out of 100 DPPC molecules is directly associated with a DIP molecule. A reversal of the observed changes upon drug association with the membrane as the DIP concentration surpasses a threshold concentration ($c_{crit} \sim 0.5$ mol%) may be explained by cooperativity in a different context, the self-aggregation of drug molecules. With its implications for the interaction of DIP with phospholipid films, this work provides a first approach to the explanation of the high sensitivity of cell membranes to piperidinopyrimidine drugs on a molecular level.

Keywords: Phospholipid monolayer; Drug–membrane interaction; Lipid headgroup conformation; Surface potential; Fluorescence microscopy; FTIR

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1. Introduction

Dipyridamole (2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-*d*]pyrimidine; DIP) and its derivatives may act as a coronary vasodilator and as a coactivator of the anti-tumor effects of other drugs through interaction with *P*-glycoprotein which is associated with multidrug resistance (MDR) developed in tumor cells [1–5]. Spectroscopic studies (absorption, fluorescence and NMR) of DIP association with membranes and with biological macromolecules [6–10] have indicated that cell membrane components are mediators of DIP interaction with biological systems. Affinity studies with micelles [6,7] and phospholipid vesicles [9,10] showed that association constants for DIP and various derivatives correlate with the hydrophobicity of the compounds. Furthermore, the association of these compounds with the membrane correlates with reported pharmaceutical effects, e.g. their inhibition of phosphate or adenosine transport across red blood cell membranes [11] and their anti-MDR activities [3–5]. Neither of these properties of the DIP family nor their inhibition of platelet coagulation or their action as anti-oxidants [12–16] have been understood on a molecular level.

While establishing a direct connection between molecular mechanisms and physiological activity requires substantial work at various levels, owing to the complexity of cellular systems, useful information has already be obtained by investigating the interaction of pharmaceutical drugs with model membranes. In this context, phospholipid Langmuir monolayers [17,18] are well-established model systems for biological membranes [19–21] as far as the lipid interface with the surrounding aqueous medium is concerned. For instance, molecular level interactions of dipalmitoylphosphatidylcholine (DPPC) with DIP and some of its derivatives that differ in their hydrophobicity have been studied by measuring lateral pressure (π) and surface potential (ΔV) isotherms [20,22] as a function of the molecular area, A, of such systems. In this work, we combine refined measurements of π–A and ΔV–A isotherms with in situ fluorescence microscopy and FTIR reflection–absorption spectroscopy (FT-IRRAS) to assess the association of DIP with DPPC monolayers particularly at low concentrations, thus demonstrating the high affinity of the drug to the membrane surface and estimating its location with respect to the lipid monolayer.

2. Experimental details

2.1. Surface pressure and surface potential measurements

L-α-1,2-Dipalmitoyl-*sn*-3-glycero-phosphatidylcholine (DPPC, purity > 99%) and DIP (chemical structure: see inset in Fig. 1) were from Sigma and used as received. Monolayer isotherms were measured on a KSV 5000 Langmuir trough equipped with a Wilhelmy plate and mounted in a class 10 000 clean room. Monolayers were prepared by cospreading[1] typically 100 μl of a premixed DPPC/DIP (0.5 g/l) solution in chloroform (Mallinkrodt, analytical grade) on subphases containing pure water (pH ~ 5.6) that was provided by a Milli-Q system coupled to a Milli-RO reverse osmosis unit (Millipore). After spreading, the solvent was allowed to evaporate for 10 min. Surface pressure vs. area per molecule (π–A) isotherms ($T = 21 \pm 1$°C) were registered while compressing the monolayer with a constant barrier speed of 10 mm/min (~ 0.1 A^2/molecule per second). The data presented here are averages of at least six measurements for each drug concentration. The surface potential of the pure and mixed DPPC monolayers were measured using the vibrating plate method (frequency $\nu = 300$ Hz) with a KSV Kelvin probe with both reference

[1] We have not taken the more customary approach of preparing a pure lipid surface monolayer and subsequently injecting the adsorbent into the subphase underneath since this would (a) lead to time dependent processes due to drug diffusion to the interface; (b) lead to inhomogeneities in the lateral distribution of the drug; and (c) would leave the final concentration of the drug at the interface quantitatively inaccessible (i.e. it might be *much* less than the amount used in the preparation whereas in the case of cospreading it is expected that the majority of drug molecules stay associated with the interface since drug solubility in water is low).

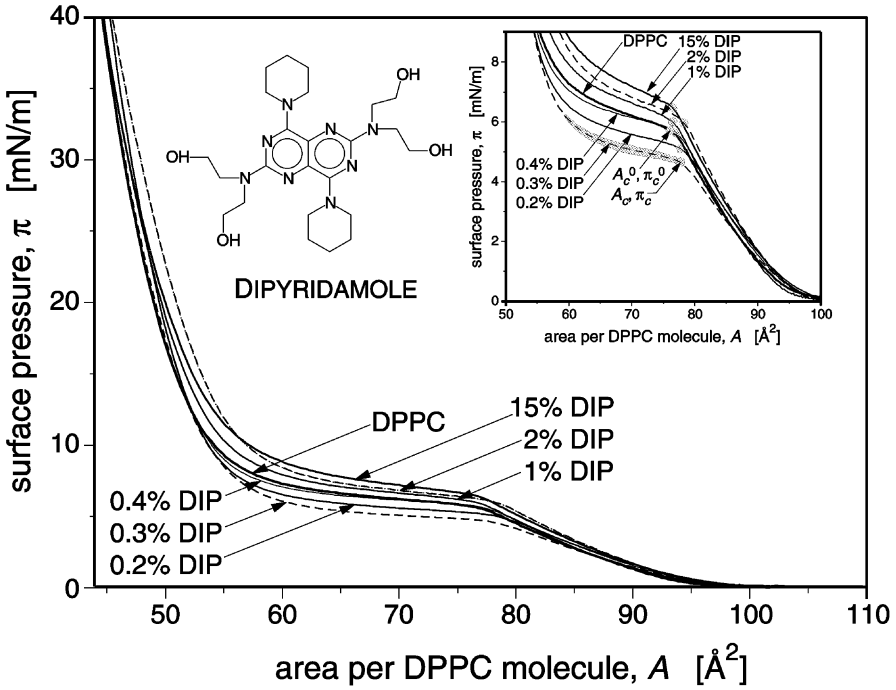

Fig. 1. Surface pressure isotherms ($T = 21$°C) of DPPC with various concentrations of DIP (chemical structure, see inset) as indicated. Data of the pure DPPC monolayer are shown as a bold line. A second inset shows a close-up view of the isotherms in the region of the onset of the LE/LC phase transition. The shaded area around the isotherm of DPPC with 0.3 mol% DIP (plateau region) visualizes the standard deviation in the data. Shaded dots indicate the phase transition onset points used to map relative changes in Fig. 2.

and vibrating plate electrodes made from platinum and the probe located approximately 2–3 mm above the water surface.

2.2. Fluorescence microscopy

Monolayers were similarly prepared as described above by cospreading chloroform (from Merck, Darmstadt, Germany) solutions of DPPC or DPPC/DIP mixtures doped with 0.1 mol% of the headgroup-labeled fluorescence probe Lissamine rhodamine B 1,2-myristoyl-*sn*-glycero-3-phosphatidylethanolamine (RhB-DMPE, Molecular Probes, Leiden, The Netherlands) on water subphases. An epifluorescence microscope (Axiotech, Carl-Zeiss Jena, Germany) was placed over a Langmuir trough of local design with a maximum area of ~ 250 cm^2. The trough is mounted on an x–y stage (Märzhäuser, Wetzlar, Germany) and may be moved relative to the position of the objective lens to select the observed area and to compensate for film drifts. For fluorescence excitation, a 50-W Hg lamp was used and excitation and emission wavelengths were selected by an appropriate beam splitter/filter combination (Zeiss filter set no. 9). The monolayer was observed from above using a $50\times$ long-distance objective lens (Epiplan 50, Carl-Zeiss Jena). Micrographs were recorded with a silicon-intensified target (SIT) video camera (C2400-08, Hamamatsu, Herrsching, Germany) connected to the microscope. Pictures were recorded directly into computer memory via an online image processor (Argus-20, Hamamatsu). The entire optical setup is placed on an active vibration isolation unit (Mod-2, JRS, Affoltern, Switzerland). Measurements were performed at $T = 21 \pm 1$°C.

2.3. FTIR measurements

FT-IRRAS measurements [23] were carried out using the external beam port of a Bio-Rad (Digilab) FTS 60A spectrometer. The IR beam is focused with a BaF_2 lens (focal length, 100 mm) and directed to the water surface with a planar Al mirror at an incident angle of $\sim 30°$. After passing a second planar mirror, the reflected beam is focused on a lN_2-cooled MCT detector using a gold-coated off-axis parabolic mirror. The sample setup consists of a custom-built thermostated Langmuir trough (8×20 cm^2) mounted on a shuttle device together with another trough containing only water. This allows the IR beam to be switched between the sample and the reference area without opening the gas-tight container that holds the entire sample configuration and the external detector. The sample compartment is purged with dry N_2. Experiments on pure DPPC and mixed DPPC/DIP monolayers at 5 mol% of the drug were performed at $T = 21 \pm 1$°C by collecting (typically) 1024 single spectra with a resolution of 4 cm^{-1}, apodized with a triangular function and Fourier-transformed with one level of zero-filling. Water subphase and vapor rotation–vibration bands were compensated by subtracting the reference spectrum from the sample spectrum.

3. Results

Fig. 1 shows isotherms ($T = 21$°C) of cospread DPPC/DIP monolayers, in which A signifies the area per DPPC molecule in the film (without accounting for any potential DIP intercalation between the lipids). It is obvious that all isotherms are quite close to the one for pure DPPC (bold trace). Subtle differences, however, are observed as shown in the inset that displays a close-up view of the isotherms near the onset of the LE/LC[2] phase transition. It is revealed that minute concentrations of DIP in the spreading solutions, as low as 0.2 mol% with respect to DPPC, condense the monolayer while any DIP concentrations larger than 0.3 mol% re-expand the monolayers such that at 0.4 mol% the same isotherm as with pure DPPC is observed. For DIP concentrations above 0.4 mol%, the monolayer is more expanded than the pure DPPC film but even at 15 mol%, the largest DIP concentration we studied, the observed pressure increase with respect to the pure DPPC film is only of the order of 1 mN/m. At lateral pressures corresponding to the LC phase, e.g. $\pi = 30$ mN/m, the values for the molecular area in all isotherms are within 3% of each other. At DIP concentrations lower than 0.1 mol% isotherms approach that of pure DPPC (results not shown). All monolayers were stable up to collapse pressures of the same magnitude (~ 46 mN/m), independent of DIP concentration. Fig. 2a visualizes the relative variations in surface pressure, $\pi_{rel} = \pi_c/\pi_c^0$, as a function of DIP concentration with respect to DPPC in the spreading solution, c_{DIP}, where π_c are the pressure values at the onset of the LE/LC phase coexistence plateaus (near $A_c^0 \sim 77$ Å^2 in the isotherms, cf. Fig. 1). The reversal of the effects for low and high drug concentrations is clearly seen.

Surface potential isotherms are presented in Fig. 3 for various DIP concentrations (as in Fig. 1, A relates to the area per DPPC molecule without accounting for DIP), and the pure DPPC data (bold trace) are consistent with published data [25–27]. As with the π–A isotherms, DIP concentrations in the range of 0.2–0.4 mol% show a significant influence on the ΔV–A isotherms while at higher DIP levels, the data resemble more closely those for the pure DPPC film. Thus, DIP levels below 0.5 mol% increase ΔV over the values observed for either the pure DPPC film or films with higher DIP concentrations at all area values along the isotherm. In particular, there is a pronounced increase of ΔV at large areas for low DIP concentrations, such that the critical area, A_c, for surface potential lift-off occurs at considerably larger values. Fig. 2b shows the relative changes in surface potential for the various DIP concentrations, taken at the onset points of the

[2] Following ref. [24], phospholipid monolayer phases are denoted as G, gaseous; LE, liquid expanded; LC, liquid condensed and SC, solid condensed.

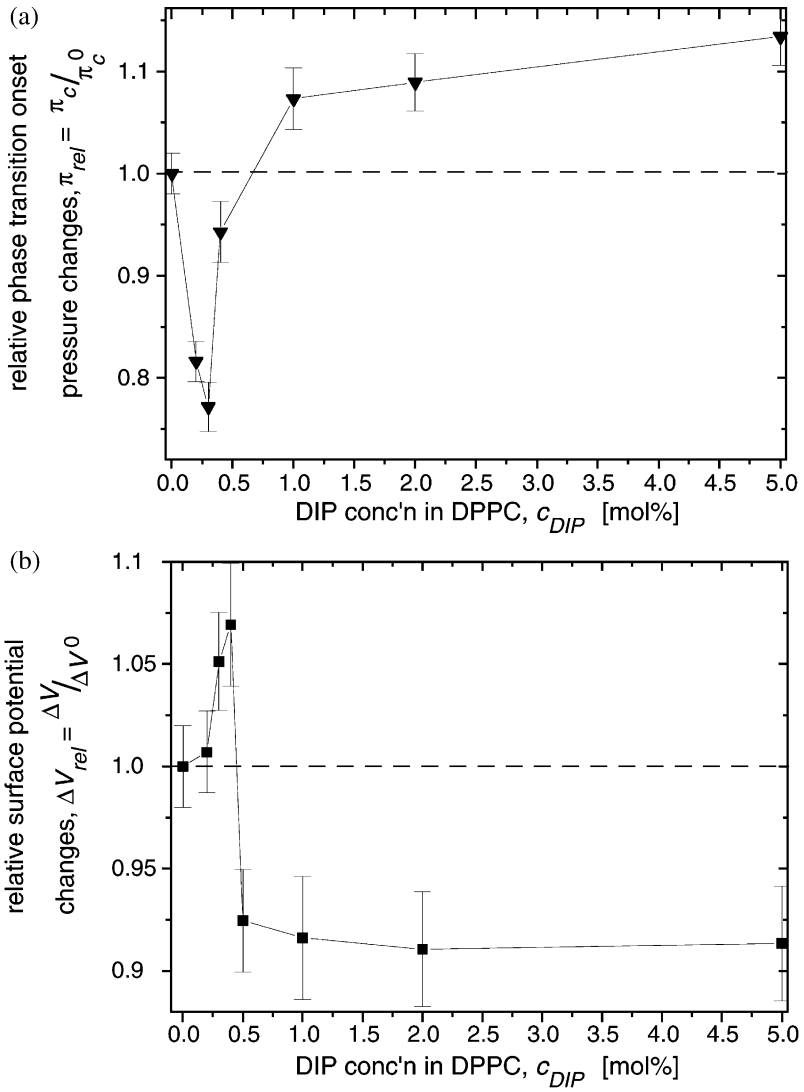

Fig. 2. (a) Relative surface pressure changes at the critical area, A_c, that marks the LE/LC phase transition onset as a function of DIP concentration with respect to DPPC. (b) Relative surface potential changes at the same areas, A_c, as obtained from the π–A isotherms as a function of DIP concentration. In both cases, (a) and (b), the respective values observed for the pure DPPC monolayers at A_c^0 have been used for the normalization. Error bars show variances of six independent runs.

plateaus in the surface pressure isotherms and normalized to the ΔV value of the pure DPPC monolayer at $A_c^0 \sim 77$ Å^2. Similarly to the π–A isotherms, a strong effect at low DIP concentrations is observed that is reversed as the DIP concentration surpasses 0.5 mol%.

Mixed DPPC/DIP monolayers of various concentrations were also investigated with fluorescence microscopy using 0.1 mol% RhB-DMPE with respect to DPPC. As discussed in the literature [28–31], LE domains are visualized in the LE/LC coexistence region of the isotherms since the LE phase dissolves the dye better than the more ordered LC phase. Figs. 4 and 5 show a comparison of domain textures for pure DPPC and mixed monolayers with various DIP concen-

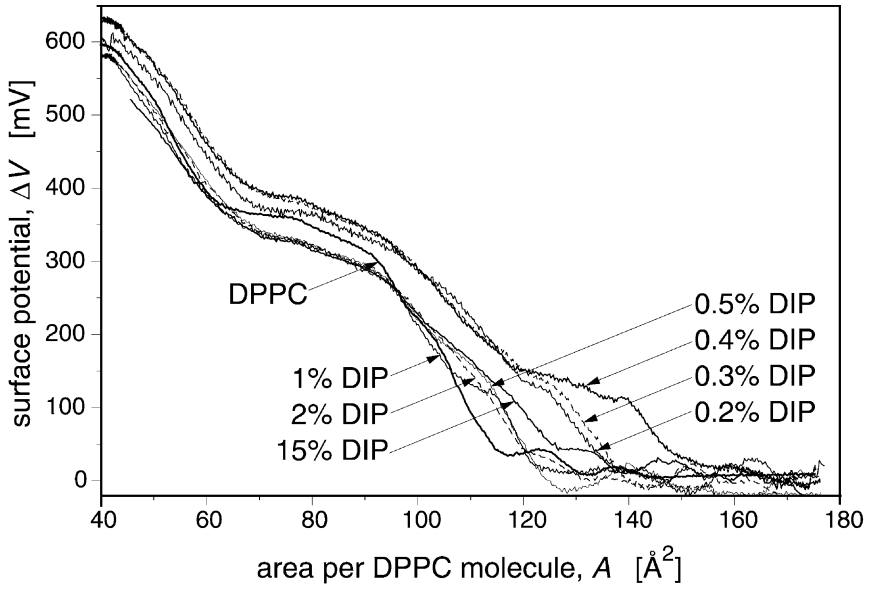

Fig. 3. Surface potential isotherms ($T = 21$°C) of DPPC with various concentrations of DIP as indicated. Data of the pure DPPC monolayer are shown as a bold line.

trations. All experiments were performed at slow compression of the monolayer (< 1 Å^2/molecule per minute), so that the observed textures are equilibrium shapes. Monolayers of the enantiomerically pure L-α-DPPC (Fig. 4a) showed characteristic textures similar to those published earlier that consist of lobed domains with curved arms due to the chirality of the phospholipid molecules [28,32]. In mixed monolayers, DIP exerts already a marked influence on the domain morphologies if included in exceedingly low concentrations. Fig. 4b–d shows micrographs of the DIP/L-α-DPPC system at 0.2 mol% DIP for various surface pressures. In comparison with the pure DPPC monolayer, Fig. 4a, it is realized that the domains grow a larger number of arms from their nucleation centers. Generally, the LE/LC boundary lines are more elongated in the presence of DIP, which leads to the formation of thin spikes as the phase transition progresses (cf. Fig. 4d, $\pi = 9.5$ mN/m). Fig. 5 displays micrographs at larger DIP concentrations (1 mol% and 5 mol% in a,b and c,d, respectively). The data at lower pressures (Fig. 5a,c) indicate a *lower* impact of the drug on the monolayer structure as its concentration is *increased*, with the domain morphologies resembling those of pure DPPC monolayers more closely at 5 mol% than at 0.2 or 1 mol%. As the phase transition proceeds in the mixed monolayers, domain structures become more elongated at all DIP concentrations. While DIP itself shows an intense fluorescence centered at $\lambda = 500$ nm it has not been possible to utilize this emission to visualize the DPPC phase separation with a suitable filter set (different from that used for the experiments with the RhB-DMPE probe). This indicates that the drug associates with both monolayer phases in a similar lateral concentration such that no contrast between the phases is afforded. It was thus necessary to use the hydrophobically substituted RhB probe for visualization.

FT-IRRAS showed that DIP exerts at best a very small influence on the phospholipid vibration modes in the mid-IR region (3000–900 cm^{-1}). Specifically, the absorption bands around 2920 and 2850 cm^{-1}, which are due to methylene stretching modes and particularly easy to observe in the experiments, are not affected by DIP association with the DPPC monolayer as shown in

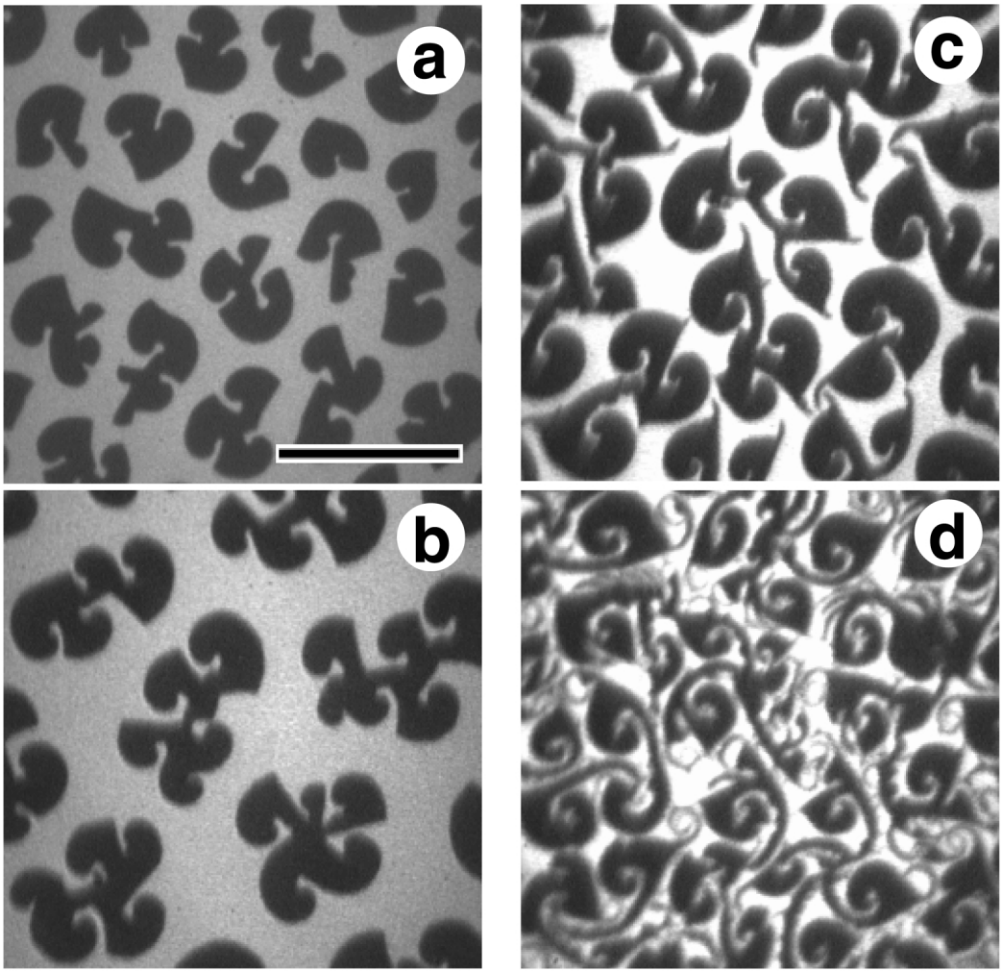

Fig. 4. Fluorescence micrographs (using 0.1 mol% of RhB-DMPE) that show the mesoscopic texture of surface monolayers of pure DPPC (a: surface pressure, $\pi = 7$ mN/m) and DPPC with 0.2 mol% of DIP (b–d: $\pi = 8$, 9 and 9.5 mN/m). The scale bar in (a) is 50 μm. $T = 21$°C.

Fig. 6a. Fig. 6b shows a comparison of band positions due to vibrations of lipid headgroup moieties [33] in the fingerprint region including the carbonyl stretching band ($\tilde{\nu}$(C=O) at 1735 cm^{-1}); the bands due to the phosphate stretching modes ($\tilde{\nu}_{as}(PO_2^{-1})$ and $\tilde{\nu}_s(PO_2^{-1})$ at 1230 cm^{-1} and 1090 cm^{-1}, respectively); and the band assigned to the C–C–N+ stretching mode in the choline ($\tilde{\nu}_s$(C–C–N+) at 975 cm^{-1}). The methylene bending mode of the acyl chains ($\delta(CH_2)$ at $\sim$1470 cm^{-1}) and a second ester absorption ($\tilde{\nu}_{as}$(C–O–C) around 1170 cm^{-1}) were poorly resolved. The only appreciable differences upon association of DIP with the interface are observed at high lateral pressure, $\pi > 30$ mN/m, for the phosphate stretch vibrations which have been shown to respond particularly sensitively to the peripheral association of adsorbent molecules [34]. Shifts of $\Delta\tilde{\nu} \sim 6$ and 2 cm^{-1} were observed, respectively, for $\tilde{\nu}_{as}(PO_2^-)$ and $\tilde{\nu}_s(PO_2^-)$ in the ordered (LC) state. Band centers of the remaining vibrational states were identical within the experimental resolution in measurements with or without DIP.

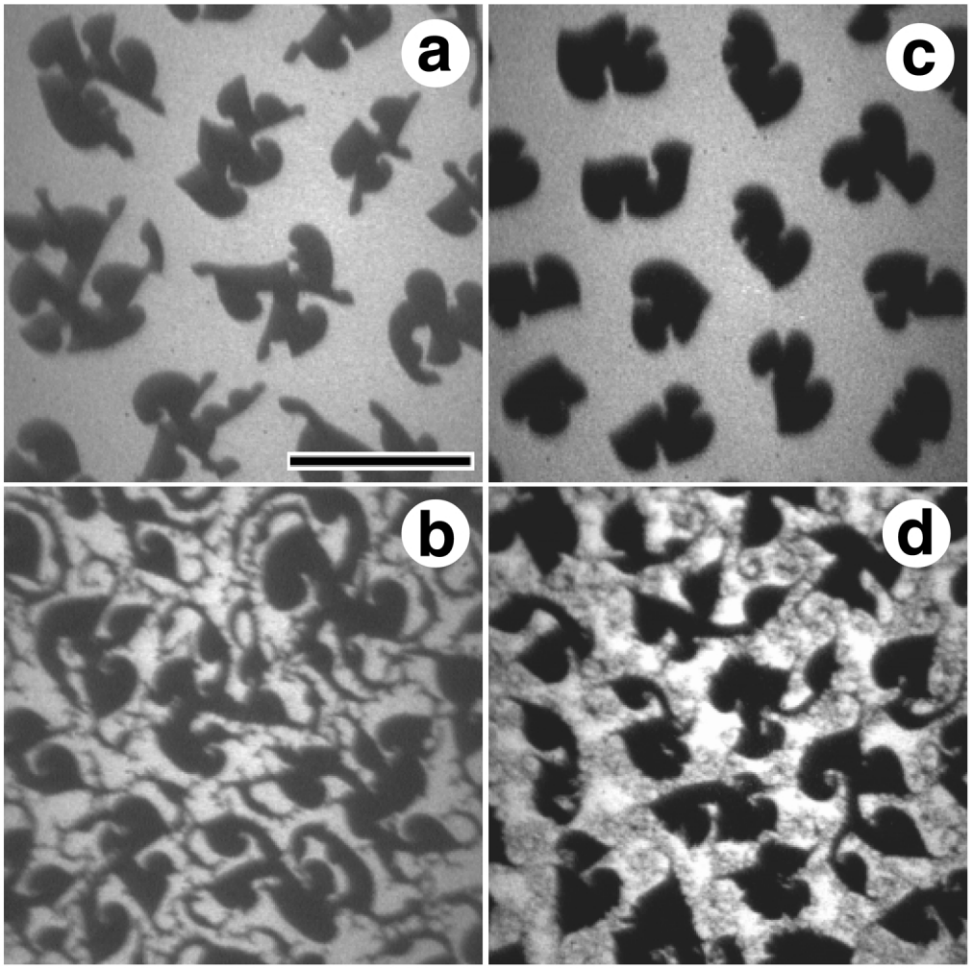

Fig. 5. Fluorescence micrographs of DPPC monolayers containing 1 mol% (a, b: $\pi = 8$ and 9.5 mN/m) and 5 mol% (c, d: $\pi = 8.5$ and 10 mN/m) DIP. The scale bar in (a) is 50 μm. $T = 21$°C.

4. Discussion

We have chosen to study the interaction of DIP with phospholipid monolayers by cospreading both substances on pure water subphases, rather than employing more conventional modes of sample preparation, e.g. the spreading pure phospholipid on a subphase presaturated with the drug or the injection of the drug into the subphase underneath a prespread phospholipid monolayer. It may be argued that the latter modes may be more relevant for investigations of physiological drug–membrane interactions. However, such preparation protocols are not adequate to study fine details of their interaction with Langmuir monolayers for the following reasons: upon dissolution in the subphase, we observed that DIP induces significant effects on phospholipid monolayers only if large amounts of the drug were used [20]. Under such conditions, however, it is impossible to distinguish bulk effects, such as changes in the ionic properties of the subphase that will affect the Debye length at the surface, from the molecular interactions of DIP with the DPPC in the surface film. Such unspecific changes may result in changes of the physico-chemical

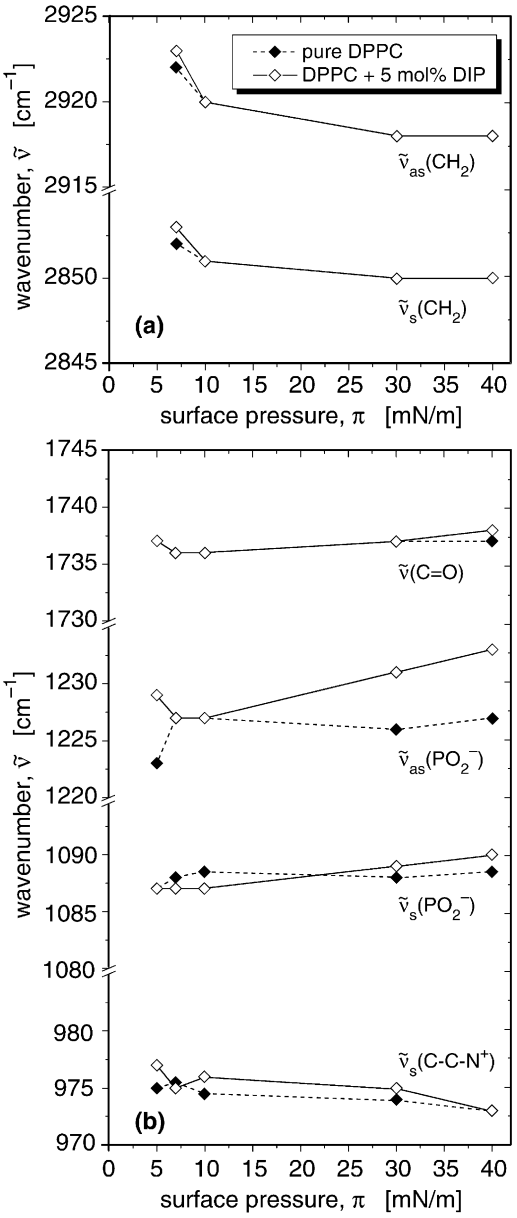

Fig. 6. Comparison of the mid-band frequencies of various lipid vibration modes, determined by FT-IRRAS, in monolayers of pure DPPC (filled symbols) and DPPC with 5 mol% DIP (empty symbols). $T = 21°C$.

properties of the system even if there was no specific interaction between DIP and DPPC. If on the other hand smaller amounts of the drug are injected, diffusion limits both the response time of the system and the homogeneity of the DIP distribution at the surface. Hence changes in monolayer properties will at best only be observed after long equilibration times, and ageing effects might compromise the significance of the experimental results. In any case, because determining the drug partition in the monolayer is not straightforward, it is difficult to assess precisely the amount of DIP that is actually interacting with the phospholipid molecules. The effects reported here could have never been observed nor would it have been possible to achieve even a semi-quantitative evaluation of the effects and to show convincingly that phospholipid headgroup reorganization occurs at drug concentrations *far below the threshold where each lipid interacts directly with one drug molecule*.

Various surface sensitive characterization methods show consistently that DIP affects physico-chemical properties of DPPC surface monolayers already at exceedingly low concentrations. Even more surprising is the fact — which was also observed with different techniques — that the effects are most pronounced in the low concentration regime while they decrease or even reverse as the DIP concentration is further increased. Both observations hint at cooperativity effects at different levels. To put the observed impact of DIP on DPPC monolayers into a perspective: If one speculates on molecular details of drug association with the surface monolayer one has to explain how one DIP per 500 DPPC may lead to the observed changes in film properties on the *macroscopic* scale. One scenario that comes to mind is an intercalation of the drug between the hydrophobic lipid chains, which might affect their local order in a cooperative mode. FTIR results showing that the methylene stretch vibrations are not affected as well as recent grazing incidence X-ray diffraction (GIXD) measurements [35], however, rule out this possibility. Moreover, previous studies on DIP interaction with surfactant micelles or DPPC vesicles also point to a location of the drug at the periphery of the lipid aggregates towards their aqueous environment, not in their hydrophobic core [6–10] — a reasonable result in view of a very low solubility of DIP in apolar solvents, such as CCl_4. There-

fore, another possibility is a peripheral association of the drug with the lipid at the interface between the subphase and the DPPC headgroups. If we approximate the geometry of a DIP molecule as a flat disk of 10 Å diameter this implies that it may interact with at most five DPPC molecules at a given time. To transpose interactions on such a level into a macroscopically measurable effect, cooperativity between the lipid molecules has to be invoked.

4.1. Regime of low DIP concentrations (< 0.5 mol%)

Surface pressure, surface potential and fluorescence microscopy data show consistently that DIP alters the state of the lipid monolayer at concentrations as low as 0.2 mol%. In this concentration regime, surface monolayers are condensed by the drug as deduced from the π–A isotherms shown in Fig. 1 and the corresponding decrease in π_{rel} at the beginning of the LE/LC coexistence region (Fig. 2a). This fact already provides some evidence against intercalation of the drug into the lipid film; rather it would be consistent with a model that has DIP interacting with more than one DPPC molecule simultaneously causing them to change conformation to pack more efficiently in the surface monolayer. However, if a DIP molecule can directly interact with five DPPC molecules and is present only in a 0.2 mol% concentration at the interface, then at most one out of 100 DPPC molecules in the film are directly affected. This coarse estimate suggests that lipids which do not directly interact with the drug might be indirectly affected via a cooperative interaction with neighboring DPPC molecules within the film. One should note that, of course, the LE/LC phase transition by itself is already a highly cooperative phenomenon involving the phospholipid molecules, as indicated, e.g. by calorimetric studies on multilamellar suspensions of DPPC where a cooperative unit size in the range of 400–600 molecules has been estimated for the pretransition (35°C) and main transition (41°C) [36]. Our results indicate that DIP at low concentrations favors formation of the LC phase by reducing the critical pressure π_c required for lipid reorganization into the phase of higher order.

A similar argument is derived from a semiquantitative analysis of the surface potential data. The conventional way to interpret the potential measured across a surface covered with an amphiphilic monolayer employs a (two- or three-) layer capacitor model [26,37–40] in which various group dipole moments on the lipid, a contribution due to the reorientation of water molecules close to the interface and the potential of the Gouy–Chapman double layer, Ψ_0, — in case of charged amphiphiles on an ionic subphase — are taken into account [40,41]:

$$\Delta V = \frac{1}{A\varepsilon_0}\left(\frac{\mu_1}{\varepsilon_1} + \frac{\mu_2}{\varepsilon_2} + \frac{\mu_3}{\varepsilon_3}\right) + \Psi_0 \qquad (1)$$

In this model, μ_1, μ_2 and μ_3 are, respectively, the contributions from the reoriented water dipoles at the monolayer interface, from dipoles at the film–water interface, and at the film–air interface. ε_i are the effective dielectric constants of the media in which the dipoles are embedded. A monolayer of the zwitterionic DPPC on pure water has no double-layer contribution [20,42]. On the DPPC molecule, the main contribution to ΔV is believed to arise from the carbonyl groups at the transition between the chains and the headgroup, as the zwitterion is surrounded by a medium with a high dielectric constant, ε_2, and is expected to be preferentially oriented parallel to the water surface [39,43]. If one evaluates, however, surface potential data of distinct zwitterionic phospholipids it is apparent that a contribution from the zwitterion may not be entirely neglected because of its extremely high dipole moment ($\sim$20 D) in comparison with other group dipole moments that are of the order of 1 D [31,42]. This implies that slight reorientation of the PC headgroup with respect to the surface normal may result in significant changes of ΔV [27,43,44] which has been used to assess the impact of adsorbent molecules on PC-containing phospholipid monolayers [27].

Peripheral adsorption of DIP in the 0.2 mol% concentration regime cannot account for the

observed changes in surface potential (on the order of 80–100 mV, cf. Fig. 3) due to a putative dipolar contribution of the drug on its own. Rather, indirect contributions due to lipid headgroup reorientation and the reorientation or displacement of water dipoles at the interface have to be invoked. Since a reorganization of the acyl chains seems of minor, if any, importance, this again points to cooperativity between neighboring DPPC molecules because if only those lipids contributed to the change that are directly involved in DIP interactions, a change in their choline dipole orientation from perfectly in-plane to perpendicular (or vice versa) could not even account for 10% of the experimentally observed change in ΔV. This conclusion can be drawn despite of the fact that a quantitative molecular level interpretation of the surface potential of phospholipid monolayers is still out of reach since the distinct dipolar contributions from several part of the headgroup cannot unambiguously be separated.

The observation that the liftoff point in the ΔV–A isotherm is changed and that surface potential values especially at low lipid surface densities are considerably increased (Fig. 3) implies that particularly DPPC molecules in the LE phase are affected in their headgroup organization. We conclude that at low lateral pressures the *mesoscopic* organization of the phase structure in the G/LE coexistence regime of DPPC, where it forms the two-dimensional analog of a fluid/gas foam [29,45], is altered through lateral condensation by the DIP molecules; this may lead to the observed changes in the liftoff point. On the *molecular* scale, the organization of the lipid headgroups and/or their association with water molecules in the LE phase is also significantly changed by adsorption of the drug.

Fluorescence microscopy shows that DIP does not preferentially adsorb to either the LE or LC phase. However, the sensitive changes which the admixture of DIP to the spreading solution causes in the domain morphology, Fig. 4, are clear evidence that the drug is present at the interface. The elongation of the domain boundaries with respect to the morphology observed with pure DPPC is the result of a reduction in line tension between the two phases and/or an increase of the repulsion between the parallel components of dipole moments located on the molecules [28,30,46]. Since a rearrangement of dipole moments on the lipid headgroups appears likely, the latter could already explain the observed effect in a qualitative manner. Furthermore, however, surface pressure and surface potential results show that drug association with the monolayer condenses the film (Figs. 1 and 2a) and that particularly the LE phase is affected by reorientation of the lipid headgroups (see discussion of the surface potential changes). This implies that the molecular order is increased in the LE phase, possibly rendering it structurally more similar to the LC phase than in the case of pure DPPC. (We note, however, that the *chain* order seems not affected by this reordering as deduced from the FTIR results.) It may thus well be that both an increase in dipolar repulsion within the monolayer *and* a decrease in line tension is responsible for the observed effects.

Finally, it should be noted that the binding constant of DIP to DPPC vesicles depends on their phase state and has been reported to be lower below the phospholipid phase transition (41°C) than above [9,10]. This hints at steric hindrance of the binding to the gel phase where the lipid is more densely packed and the lipid headgroup may not be sufficiently flexible to accommodate the drug. Furthermore, electron paramagnetic resonance studies using the nitroxide spin labels, 5- or 16-doxyl stearate, showed a very small effect of DIP on DPPC vesicles [47]. This effect was larger for 5-doxyl stearate which is located closer to the membrane interface. These results are consistent with the data reported here, which show that the DIP affects DPPC more pronouncedly in the regime of the LE/LC transition than in the pure LC phase at higher surface pressures — which is the corresponding state to that of vesicle membranes at the lower temperatures.

4.2. Higher DIP concentrations

Particular attention is due to the concentration dependence of the drug effects on the phospholipid monolayers. All three investigated quantities

— surface pressure, surface potential and domain morphology — point to the conclusion that DIP is present at the interface in a configuration that interacts strongly with the lipid at exceedingly low concentration. However, upon increasing the drug concentration above a certain, low threshold ($c_{crit} \sim 0.5$ mol%) these effects do not just saturate but are revoked or even reversed (monolayer *expansion* instead of *condensation*). This must be attributed to a cooperative effect that acts on an entirely different level than the one discussed in the previous section with respect to the response of the lipids to drug association: We postulate that DIP aggregates at the interface upon surpassing c_{crit}. For the discussion of such a self-aggregation of the drug, one has to realize that its equivalent bulk concentration at the interface is extremely high: If the DIP molecules are entirely confined within an interface layer, 50 Å thick, a surface concentration of 0.2 mol% with respect to lipid is equivalent to a bulk concentration of ~ 1 mmol/l. In aqueous solution, the solubility of DIP is only ~ 30 μmol/l [7].

The reversal of the low concentration effects implies that the monomer concentration of the drug in its active state at the interface is *reduced* below the concentration for which these association effects have been observed, i.e. below 0.2 mol%. Aggregation may well account for such a concentration behavior. The aggregated state may be either membrane-bound or may dissociate from the membrane: In any case its interaction with lipid molecules is so weak that macroscopic effects are no longer expected. DIP aggregation was in fact suggested to explain the pressure–area isotherms in previous work [20], where only DIP concentrations above 1 mol% were used.

4.3. Molecular scale interpretation of DIP interactions with DPPC monolayers

While FTIR spectroscopy is not as sensitive a tool to characterize molecularly thin interface films as are the other techniques, it is capable of providing information on specific chemical moieties. A particularly sensitive measure for conformational changes of the acyl chains are the methylene stretch vibrations. The fingerprint region between 1800 and 900 cm^{-1} contains information on various vibrations located on the phospholipid headgroups. Neither of the these bands (with the exception of $\nu(PO_2^-)$) was observed to change upon DIP association with the lipid. The methylene stretch vibrations are particularly easy to assess and the comparison (Fig. 6a) between the pure DPPC and the DPPC/DIP system shows that frequency changes upon compression of the monolayers are indistinguishable within experimental error for the two cases. We conclude that DIP does not intercalate between the lipid's acyl chain upon association with the surface monolayer. This is also in agreement with the poor solubility of DIP in apolar solvents, which is even lower than that in water. Similarly one may rule out major reorganization of the carbonyl region at the periphery between the acyl chains and the lipid's headgroups upon DIP interaction with DPPC. The C=O vibration has been shown to be particularly sensitive to hydration changes [48] which may induce large frequency shifts. In the work reported here, the center frequencies of these bands were observed to be insensitive to DIP association within experimental error. Of the accessible fingerprint vibration bands, only the phosphate absorptions, $\nu(PO_2^-)$, were found to shift upon DIP association with DPPC by (maximally) 7 and 2 cm^{-1} for the asymmetric and symmetric modes, respectively. The fact that shifts to higher energies are observed suggests an increase of the force constant D (in a relation for the frequency, $\nu \propto \sqrt{D/\mu}$ with μ being the reduced mass of the oscillator in a harmonic approximation) indicative of an increased strength of the P–O bond. An explanation for such an increase might be a change in the molecular conformation that transfers the phosphate group to a less polar environment where it is involved into hydrogen bonding to a lesser extent.

What is a likely mechanism of the coupling of DIP to the lipid monolayer? — From the chemical structure of the drug, inset in Fig. 1, and pH titrations [7] it is inferred that both hydrogen bonding and electrostatic interactions might be important. DIP accepts a proton at one of its tertiary nitrogens in a slightly acidic medium (pK 5.8 [7]), just close to the pH of the aqueous

subphase in the experiments reported here (since ambient CO_2 dissolves into the subphase water), resulting in a positive charge located on the molecule. On the other hand, the terminal hydroxy groups at the periphery of the molecule are potential hydrogen bond donors. This explains why the drug is so strongly interacting with the phospholipid headgroup in the disordered (LE) state where the headgroups are preferentially aligned parallel to the interface [49–51] such that the phosphate is accessible from the aqueous phase. We suggest that the positive charge on the DIP molecule locks to the negatively charged phosphate of one DPPC while its ethanolamine hydroxy groups form hydrogen bonds with phosphates on up to four neighboring lipids. This may lead to a strong coupling of the drug to the lipid monolayer which then explains the observation of DIP-induced changes of monolayer properties already at exceedingly low drug concentrations. Electrostatic interactions — attraction between the core of the DIP molecule and the phosphate on the DPPC as well as repulsion between DIP and the choline — will reorient the headgroup dipole moment μ_2 [cf. Eq. (1)] such that its projection on the surface normal, and thus the surface potential, is expected to increase: The negatively charged phosphate is drawn toward the subphase while the positively charged choline is pushed toward the hydrophobic compartment.

5. Conclusions

DIP interacts strongly with DPPC in surface monolayers on water and changes characteristic monolayer properties at exceedingly small concentrations, as low as 0.2 mol.% with respect to the lipid.[3] Its impact is, however, revoked as the drug concentrations are increased to 0.5 mol.% and even reversed beyond that. IR spectroscopy indicated that the drug interacts peripherally with the zwitterionic lipid headgroups but does not deeply — if at all — intercalate between the lipids. We propose that the DIP molecule associates with up to five lipid molecules at a time via electrostatic and hydrogen bonding interactions. The resulting reorganization of the lipid is highly cooperative, such that the reported macroscopic effects occur at concentrations in which one DIP molecule interacts only with one out of 100 lipid molecules within the monolayer. The effect of DIP is more pronounced within the LE/LC coexistence region where the cooperative phase transition of phospholipid molecules occur. In the condensed state the effects are smaller, consistent with results for lipid vesicles. Self-aggregation of the drug molecules at the interface may lead to the observed reduction — or inversion — of changes in monolayer properties as the DIP concentration is increased beyond 0.5 mol.%, which corresponds to a bulk concentration in excess of 1 mM.

[3] We emphasize that the experimental situation deriving from the preparation protocol in which drug and phospholipid molecules are co-spread at the aqueous interface is likely to differ from a situation in which the drug is brought to the interface from the aqueous compartment by diffusion.

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