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Modulation of tetracycline–phospholipid interactions by tuning of pH at the water–air interface

B. Mecheri[a,*], F. Gambinossi[a], M. Nocentini[b], M. Puggelli[a], G. Caminati[a,*]

[a] *Dipartimento di Chimica, Università di Firenze-Via della Lastruccia, 3, Sesto Fiorentino 50019 Florence, Italy*
[b] *Dipartimento di Firenze-Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Via di Castelpulci 41, 50010 San Martino alla Palma, Florence, Italy*

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Abstract

This paper is part of a systematic study of the interactions of tetracycline antibiotics with phospholipid monolayers at the water–air interface. Tetracyclines are widespread antibiotics that undergo a series of protonation equilibria in solution, depending on the pH. The surface activity of tetracyclines was determined by means of surface tension measurements for three different systems, i.e. water, TRIS and McIlvaine-EDTA buffer. Surface pressure–molecular area and surface potential–molecular area isotherms were acquired for dipalmitoylphosphatidic acid monolayers on TRIS buffer (pH = 7.0) and McIlvaine-EDTA buffer (pH = 4.0) solution as a function of tetracycline concentration in the subphase. Comparative analysis of surface potential data, with the molecular dipole moment of tetracycline obtained from semiempirical calculations, provided information on the orientation of tetracycline at the interface. Surface pressure measurements as a function of monolayer compression were described, applying either a continuous partition model or Langmuir adsorption isotherms. The results obtained in the case of buffer solutions were compared to those obtained for tetracycline in water subphases. The analysis of the results indicated that electrostatic interactions dictate the migration of tetracycline to the monolayer interface. Penetration of the molecule to the lipophilic portion of the monolayer was unlikely, especially at high surface pressures.

The results showed that stronger interactions are established between the zwitterionic tetracycline and the deprotonated phosphatidic group in TRIS buffer solution; in this case, tetracycline binds at the monolayer interface following a Langmuir type adsorption. In the case of water, where the monodeprotonated acid and the tetracycline zwitterions are the only species involved, the data can be described by continuous partition of tetracycline between interfacial and bulk phases. The same holds for McIlvaine-EDTA buffer subphases, although the high concentrations of citrate ions in this buffer competitively interfere with tetracycline association at the monolayer interface.

Keywords: Antibiotics; Dipalmitoylphosphatidic acid; Surface pressure; Surface potential; Langmuir adsorption; Partition coefficient

1. Introduction

Interactions between antibiotics and models of biological membranes, such as phospholipid monolayers at the air–water interface, have long attracted the attention of the scientific community [1,2]. Such investigations are important in two respects: to elucidate the molecular basis of the mechanism of action, and to provide the physico-chemical basis for the fabrication of sensors for the specific drug. We focused our attention on a particular class of antibiotics: the tetracycline family. The widespread use of these molecules in veterinary medicine is creating increasingly serious risks for the unwary consumer of animal-derived products such as milk and meat.

Tetracyclines are antimicrobial compounds that induce allergic syndromes and strain resistance to antibiotics [3]; for this reason, extremely low limits are imposed by international legislation for tetracycline concentrations in food. It is generally believed that tetracycline action involves penetration through biological membrane, but little is known about the details of such processes [4]. This paper is part of a systematic study of the mechanism of interaction of tetracycline antibiotics with phospholipid monolayers at the water–air interface. In the first part [5], we studied the spreading monolayers of three typical phospholipid constituents of natural membranes in the presence of tetracycline in the subphase. We studied monolayers of dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanol-

* Corresponding authors.
E-mail addresses: mecheri@csgi.unifi.it (B. Mecheri), caminati@unifi.it (G. Caminati).

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amine (DPPE) and dipalmitoylphosphatidic acid (DPPA) in the presence of increasing amounts of Tetracycline (TC) in the subphase. The analysis of surface pressure and surface potential data showed that migration of tetracycline towards the interface is promoted in the presence of the phospholipid, basically due to electrostatic reasons. We observed higher interactions in the series DPPE < DPPC < DPPA; nevertheless, detailed information on the mechanism of such interaction could not be obtained because of the presence of multiple protonation equilibria of tetracycline in water solution. The present paper deals with tetracycline in buffer solution where the distribution of the TC species is fine-tuned by a judicious selection of the pH value of the subphase. We selected two specific buffer solutions: TRIS buffer (pH = 7.0) and McIlvaine-EDTA buffer (pH = 4.0) in analogy with current analytical studies for tetracycline determination in food-derived fluids [6]. The results were correlated with surface activity measurements of tetracycline at the water–air interface as a function of the pH of the subphase. The experimental data were interpreted on the basis of two different models: equilibrium partition of tetracycline, and Langmuir adsorption isotherms. Surface potential data were analysed and compared with the electrostatic potential distribution and the molecular dipole moment of tetracycline obtained from semiempirical calculation in order to determine the orientation of tetracycline at the interface.

This study may well provide not only further insight into the mechanism of action of tetracycline antibiotics but also a key indication for the fabrication of mimetic sensors for such drugs.

2. Experimental

2.1. Materials

Tetracycline hydrochloride (TC) and dipalmitoylphosphatidic acid (DPPA) were supplied by Sigma (purity > 99%); the chemical structures of TC and DPPA are reported in Fig. 1.

Chloroform, supplied by Aldrich, was used as spreading solvent. Phospholipid concentrations of 1×10^{-3} M were typically used for spreading solutions, unless otherwise stated in the text. The water used as subphase was obtained from a Milli-RO coupled with a Milli-Q set-up (Millipore): resistivity 18.2 MΩ cm, pH 5.6 at 20 °C. TRIS buffer (pH = 7.0; $C = 0.05$ M) was prepared using Tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) (assay > 99%) and Tris(hydroxymethyl)aminomethane (Tris-buffer) (assay > 99.8%,) purchased from Fluka. McIlvaine-EDTA buffer (pH = 4.0, $C = 0.191$ M) was prepared using disodium hydrogen phosphate dihydrate (assay > 99%) obtained from Fluka and citric acid monohydrate (assay > 99.8%) supplied by Baker, adding ethylenediaminetetraacetic acid disodium salt (EDTA) (assay > 99%) purchased from Fluka.

2.2. Methods

Surface tension, γ, measurements were obtained with the Du Nouy method using a Mettler balance with a platinum ring (Ø 2 cm) immersed in a trough containing the solution to be examined. Surface tension was measured as a function of TC concentration in the subphase; [TC] was varied by standard addition of aliquots of a stock solution of tetracycline ($[TC]_{stock} = 10^{-2}$ M) and 30 min were allowed for equilibration before each γ measurement.

Surface pressure–surface area, π–A, isotherms were obtained with a Lauda Filmwaage FW2 (Lauda, Germany) by discontinuous compression; the compression rate was 8 Å^2 molecule^{-1} min^{-1} and three π values were recorded for each surface area with a time interval of 30 s between the measurements. Thirty minutes was allowed for solvent evaporation and TC equilibration at the interface prior compression. All the spreading isotherms shown in this paper are the average of at least three curves to ensure reproducibility. A Basic-20 pH-meter from Crison was used

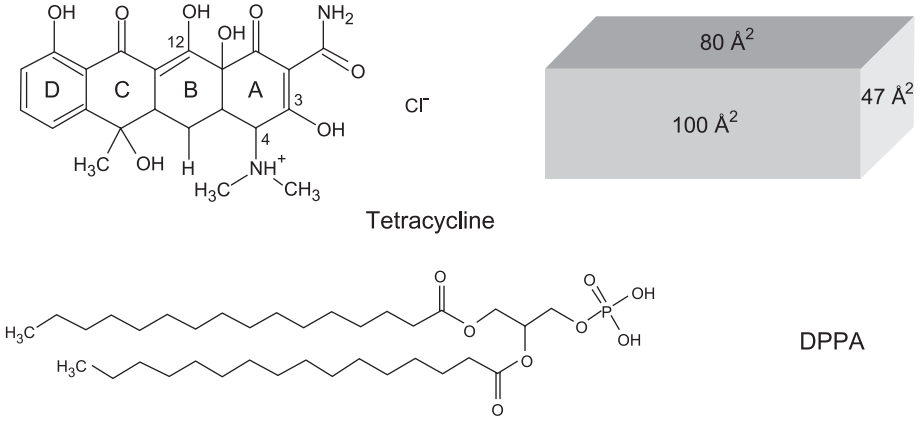

Fig. 1. Chemical structure of tetracycline and DPPA.

to determine the pH of the solutions as a function of tetracycline concentration.

Surface potential–surface area, ΔV–A, measurements were obtained using ^{241}Am ionising electrodes by means of an apparatus, previously described [7,8], assembled in the Department of Chemistry (Florence).

Spreading and adsorption isotherms were obtained at 20 °C using a Haake thermostat with water circulation bath. The accuracy of γ was ± 0.1 mN m^{-1}, of π was ± 0.1 mN m^{-1}, of A was ± 0.5 Å^2 molecule^{-1} and of ΔV was ± 10 mV.

Semi-empirical calculations (PM3, AM1) [9,10] were run using the software HyperChem 5.1 (HyperCube, USA).

3. Results and discussion

3.1. Adsorption isotherms of tetracycline at water–air interface

Adsorption isotherms of TC at liquid/air interface, $\gamma - \log$ [TC] at 20 °C, are reported in Fig. 2 for buffer and aqueous solutions.

Measurements were possible only for concentrations below the solubility limit of tetracycline. The solubility limit of tetracycline is different in water, TRIS buffer and McIlvaine-EDTA buffer and, in particular, we used tetracycline concentrations below 3.5×10^{-3} M for buffer solutions and [TC] below 10^{-2} M in the case of water solutions. Above these concentrations, we observed precipitation of tetracycline.

Comparison between the three curves shows that surface activity of tetracycline is higher in McIlvaine-EDTA buffer, where a maximum decrease of 16 mN m^{-1} in surface tension is recorded. We also observed that surface tension for McIlvaine-EDTA solution is higher than water surface tension in the absence of tetracycline, suggesting that citrate ions at this high ionic strength accumulate at the interface, inducing a reorganization of the interfacial layer. A decrease in surface tension of 7 mN m^{-1} is observed for tetracycline both in TRIS buffer and in water, but $\gamma - \log$ [TC] curves shift towards higher concentrations when passing from TRIS buffer to water. In the latter case, we observed similar behaviour to that recorded at 25 °C and reported in a previous paper [5], even if the higher solubility limit at 25 °C allowed us to explore a wider tetracycline concentration range.

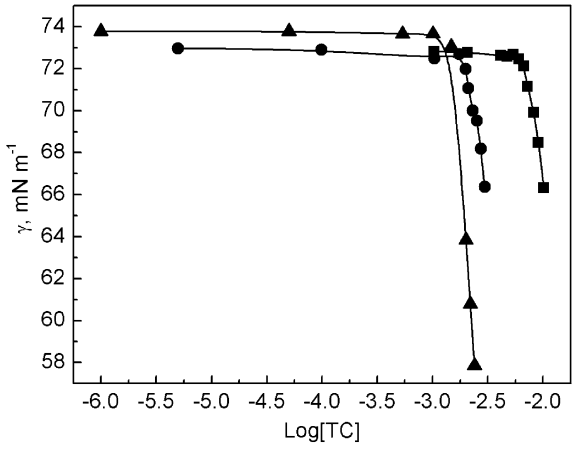

Fig. 2. Surface tension isotherms at 20 °C for TC for water (squares), TRIS buffer (circles) and McIlvaine-EDTA buffer (upward triangles).

Table 1
Surface excess, Γ_{TC}, molecular area, A_{TC}, and predominant species of tetracycline in solution at different pH computed from Gibbs adsorption isotherm; $n = 1$ for nonionic compounds and $n = 2$ for singly charged substances

	n	Γ_{TC}, mol^{-1} cm^{-2}	A_{TC}, Å^2 molecule^{-1}	TC species (%)	pH
Water	2	2.76×10^{-10}	60	H_4TC^+ (85)	2.5
TRIS buffer	1	5.67×10^{-10}	31	H_3TC (99)	5.5
McIlvaine-EDTA buffer	1	1.28×10^{-10}	13	H_3TC (85)	4.0

We applied the Gibbs adsorption isotherm (Eq. (1)) to the linear portions of the $\gamma - \log$ [TC] curve to determine the surface excess, Γ_{TC}, and the molecular area, A_{TC}, occupied by tetracycline at the interface:

$$\Gamma_{TC} = -\frac{C_{TC}}{nRT} \cdot \left(\frac{\partial \gamma}{\partial C_{TC}}\right)_T \quad (1)$$

where C_{TC} is the bulk concentration of tetracycline, $n = 1$ for nonionic compounds and $n = 2$ for singly charged substances [11]. The results are reported in Table 1.

As previously reported [5,12,13], tetracycline undergoes several protonation equilibria in solution, therefore the choice of the n value is dictated by the pH of the solution in the concentration range considered. The pKs, determined either by potentiometric or spectroscopic methods, are p$K_1 = 3$–4 (OH3 group in the A ring), p$K_2 = 7$–8 (OH12 group on the B ring), p$K_3 = 8.8$–9.8 (dimethylamino group in the A ring) [12–15]. The protonation equilibria of TC molecule also induce a change in molecular conformation: tetracycline in acidic to neutral solution adopts a twisted conformation in order to relieve the steric crowding between the protonated nitrogen on the dimethylamino group, NH4, and the OH12. Above pH 8, the conformational equilibrium is shifted towards the extended conformation. In the concentration range studied throughout this paper (pH < 8), we may safely assume that tetracycline adopts the twisted conformation [12,13,16]. The pH of TC solutions is expected to change with concentration. The experimentally measured pH of the solutions used in the experiments are reported in Fig. 3a where it is clear that high concentrations of tetracycline (log[TC] > – 2.5) provoke a decrease in pH also in TRIS buffer solution.

The decrease in pH with concentration is almost linear in the case of water; pH remains constant only for McIlvaine-EDTA buffer. We computed the distribution of the different TC species as described in a previous paper [5]. The results reported in Fig. 3b show that only the totally protonated

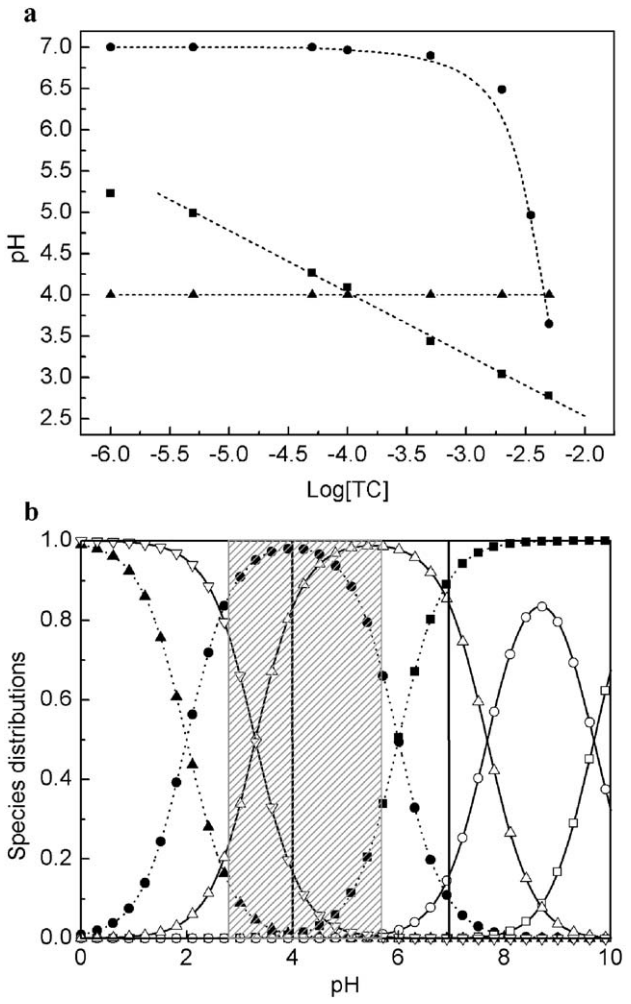

Fig. 3. (a) pH vs. log [TC] for water (squares), TRIS buffer (circles) and McIlvaine-EDTA buffer (upward triangles). (b) Species distributions of tetracycline and DPPA as a function of pH. H_4TC^+ (empty downward triangles), H_3TC (empty upward triangles), H_2TC^- (empty circles), HTC^{2-} (empty squares), H_2DPPA (filled upward triangles), $HDPPA^-$ (filled circles), $DPPA^{2-}$ (filled squares). The shaded region is the pH range for solutions of tetracycline in water. Solid lines: pH for TRIS buffer and McIlvaine-EDTA buffer.

form, H_4TC^+, and the neutral form, H_3TC, are present in significant population in the pH range examined, with a negligible fraction of the negatively charged molecule, H_2TC^-, in TRIS buffer. The equilibrium among the different species moves towards the protonated form with increasing [TC].

Γ_{TC} data reported in Table 1 suggest that the neutral form, H_3TC, has a higher tendency to adsorb at the interface compared to H_4TC^+. The lower value of Γ_{TC} obtained in the case of McIlvaine-EDTA buffer can be ascribed to the competitive presence of citrate ions at the interface. Molecular areas for tetracycline in the adsorption layer were obtained from Γ_{TC} values; the results reported in Table 1 were compared with the theoretical predictions for the molecule. Energy minimisation of the molecular structure in vacuo, performed with semi-empirical methods, provided a cross-sectional area of 97 $Å^2$ for an orientation of the plane of the BCD rings parallel to the interface. In particular, for the tetracycline/water system we assumed that only the protonated form H_4TC^+ is present (see Table 1) in this concentration range, disregarding the 15% of population present in the neutral form, and we obtained $A_{TC} = 60$ $Å^2$, using $n = 2$ in Eq. (1). Lower molecular area values than expected from geometrical consideration could hence be explained by this approximation that disregards the presence of both species at the interface. A similar result was also found in a previous work at 25 °C [5] where we also extended the measurements to higher tetracycline concentrations, i.e. where only H_4TC^+ is present, finding molecular areas in good agreement with geometry optimization.

In the case of TRIS buffer, we selected $n = 1$ in Eq. (1) since the predominant species is H_3TC. As reported in Table 1, the area values computed for buffer solutions are smaller than in the case of water solutions, that is to say $A_{TC} = 31$ $Å^2$. These small molecular area values may be explained by the formation of TC aggregates at the interface; in this case, Gibbs equation for the monomolecular adsorption layer is no longer valid. TC aggregation behaviour could be favoured by the presence of stacking interactions between the neutral H_3TC molecules; a similar behaviour is reported for tetracycline also by other authors [17]. On the contrary, in the case of the charged species (H_4TC^+), such interactions would be greatly hindered by electrostatic repulsion.

Regarding McIlvaine-EDTA buffer, the predominant species is the neutral one and, assuming $n = 1$ in the Eq. (1), we obtained a molecular area value of 13 $Å^2$. This low and unrealistic value can be explained considering that the application of the Eq. (1) is affected not only by the assumption $n = 1$ (the 15% of population present in the charged form was disregarded) but also by a competitive adsorption process of citrate ions to the interface that hinders the TC adsorption (see below).

3.2. Surface pressure–molecular area, π–A, and surface potential–molecular area, ΔV–A, isotherms of DPPA monolayers on subphases containing tetracyclines

π–A and ΔV–A isotherms of DPPA on TRIS buffer and on McIlvaine-EDTA buffer are reported in Figs. 4 and 6, respectively. For the sake of clarity, we also report in Fig. 5 typical isotherms obtained for DPPA on the tetracycline/water subphase, discussed at greater length in a previous work [5]. The tetracycline content in the subphase varies in the range 1×10^{-6}–5×10^{-3} M; surface tension results reported above show that tetracycline does not contribute to surface pressure in this concentration range, except for McIlvaine–EDTA buffer subphase at high tetracycline concentrations. In the same figures the behaviour of surface potential as a function of the phospholipid molecular area is also described for some typical tetracycline concentrations.

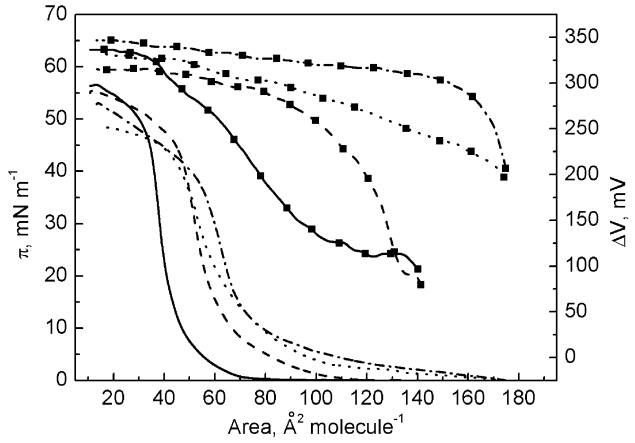

Fig. 4. Surface pressure (lines) and surface potential (lines and symbols) vs. molecular areas for DPPA at 20 °C on subphases containing tetracycline in TRIS buffer (pH = 7.0) at different TC concentrations: 1×10^{-6} M (—); 5×10^{-5} M (- - - - -); 1×10^{-4} M (·····); 3.5×10^{-3} M (· – · – ·).

In Table 2 we report the main parameters obtained from the experimental isotherms, that is to say the limiting molecular area A_0 and the maximum value of surface compressional modulus, C_S^{-1}, for the lowest and the highest tetracycline concentrations.

A_0 was determined extrapolating the linear portion in the condensed region of the isotherm to zero surface pressure, and C_S^{-1} was computed from Eq. (2) [18]:

$$C_S^{-1} = -A \cdot \left(\frac{\partial \pi}{\partial A}\right)_T. \quad (2)$$

The collapse surface pressure (π_C) values of DPPA monolayers decrease as a function of tetracycline concentration in the case of both water–tetracycline and TRIS buffer–tetracycline systems, whereas regarding McIlvaine-EDTA buffer, they remain almost constant around 48 mN m^{-1}.

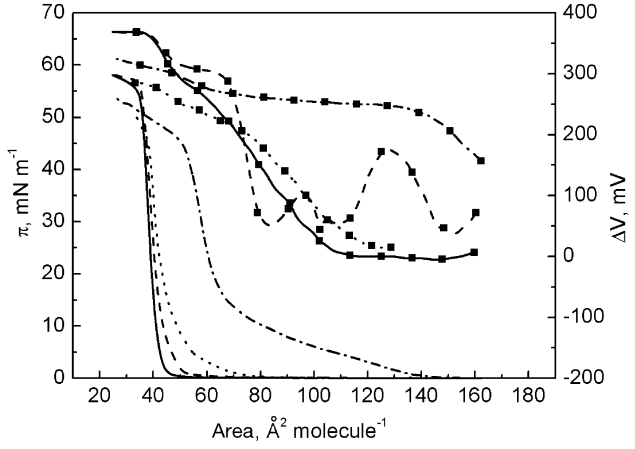

Fig. 5. Surface pressure (lines) and surface potential (lines and symbols) vs. molecular areas for DPPA at 20 °C on water subphases containing tetracycline at different concentration: 1×10^{-6} M (—); 5×10^{-5} M (- - - - -); 1×10^{-4} M (·····); 5×10^{-3} M (· – · – ·).

Table 2
Limiting molecular area, A_0, and surface compressional modulus, C_S^{-1}, values extracted from the π–A isotherms

	A_0, Å^2 molecule^{-1}			C_S^{-1}, mN m^{-1}		
[TC], M	0	1×10^{-6}	5×10^{-3}	0	1×10^{-6}	5×10^{-3}
Water	40	41	67	397	356	138
TRIS buffer	45	46	104	198	166	79
McIlvaine-EDTA buffer	64	70	77	74	63	91

The monolayer features for DPPA in the absence of tetracycline are in good agreement with the results of previous works dealing with DPPA monolayers [7,19,20] on water, whereas little is known on the specific effect of TRIS or McIlvaine-EDTA buffer. Analogously, ΔV values obtained for DPPA on water compare well with the literature reports on the same compounds at different pHs [21,22]. It is evident from the figures and from the parameters reported in Table 2 that both the tetracycline and the buffer system affect the isotherms of DPPA to different extents. The three systems will be examined individually.

3.3. DPPA monolayer on tetracycline/TRIS buffer subphase

DPPA isotherms on TRIS buffer (pH = 7.0) subphases containing tetracycline are shown in Fig. 4 for $1 \times 10^{-6} \leq [TC] \leq 5 \times 10^{-3}$ M. The isotherms move toward higher molecular areas with increasing [TC]: for $[TC] > 5 \times 10^{-6}$ M, a new liquid-expanded phase appears in the isotherms at low surface densities (A>120 Å^2 molecule^{-1}). The transition to a more condensed phase is reflected in a plateau centred at $\pi = 5$ mN m^{-1}. The expansion effect due to tetracycline, although smaller in the condensed phase, is clearly shown by the variation of the limiting areas that change from 45 Å^2 per molecule for DPPA on TRIS buffer to 104 Å^2 per molecule for DPPA on TRIS buffer at the highest TC concentration examined. Moreover, surface compressional modulus values as well as collapse surface pressures decrease with increasing [TC] (see Table 2), reflecting the presence of more expanded phases and excluding expulsion of tetracycline from the interface. The presence of tetracycline in the subphase causes, in the expanded phase, an appreciable change of the shape of the ΔV isotherms that move towards higher surface potential values with increasing [TC]. Conversely, ΔV is almost unaffected by the presence of TC in the subphase in the condensed monolayer phases. ΔV–A isotherm of DPPA for $[TC] = 5 \times 10^{-3}$ M (not shown here) exhibits the presence of spikes, due to a large monolayer heterogeneity, at both high and low surface densities.

3.4. DPPA monolayers on tetracycline/water subphase

As already discussed [5], π–A and ΔV–A isotherms of DPPA on the water subphase, as a function of tetracycline concentration, show a substantial displacement to higher surface area values already at low [TC] (see Fig. 5). The

increase in limiting areas in Table 2 is significant at [TC] = 10^{-4} M and A_0 reaches a maximum value, for the highest tetracycline concentration, of 67 Å^2, which corresponds to an increase of about 27 Å^2 per DPPA molecule. Furthermore, collapse surface pressure remains almost constant for [TC] ≤ 2 mM; otherwise, π_c decreases as a function of TC content. As in the case of TRIS buffer subphases, the shape of the isotherms starts changing at TC concentrations as low as 1×10^{-4} M, and a liquid expanded phase appears at low DPPA surface density. In this TC concentration range, the monolayer also remains more expanded upon further compression. The progressive monolayer expansion with the increase of [TC] is also evident from the change of 218 mN m^{-1} for the maximum C_S^{-1} in the tetracycline concentration range 1×10^{-4}–5×10^{-3} M. Migration of tetracycline towards the interface is also reflected in the behaviour of the surface potential as a function of monolayer compression. ΔV varies in both the expanded and condensed phases of the monolayer, when compared to tetracycline-free subphases, even if this effect is more evident in the liquid-expanded phase.

3.5. DPPA on tetracycline/McIlvaine-EDTA buffer

Unlike in the other two systems, DPPA isotherms on McIlvaine-EDTA buffer subphase (pH = 4.0), containing tetracycline in the concentration range 1×10^{-6}–5×10^{-3} M, show a very slight displacement of molecular areas with the increase of [TC], as described in Fig. 6. The limiting molecular area varies in the range 64–77 Å^2 per molecule in the tetracycline concentration range examined whereas π_C values remain constant in the range of 41–47 mN m^{-1}, suggesting that incorporation of tetracycline in the monolayer is unlikely at maximum monolayer packing. Low C_S^{-1} values (60–90 mN m^{-1}) indicate the presence of more expanded phases but this also holds for the tetracycline-free subphase: with McIlvaine-EDTA buffer buffer, we observe a significant shift towards larger areas of DPPA isotherms also in the absence of tetracycline.

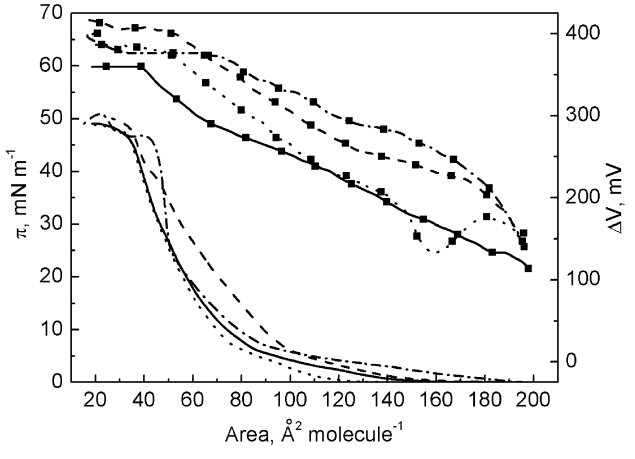

Fig. 6. Surface pressure (lines) and surface potential (lines and symbols) vs. molecular areas for DPPA at 20 °C on subphases containing tetracycline in McIlvaine-EDTA buffer (pH = 4.0) at different TC concentration: 1×10^{-6} M (—); 1×10^{-4} M (- - - -); 5×10^{-4} M (·····); 2×10^{-3} M (· — · — ·).

It is well known from the literature [20–22] that changes in the pH of the subphase affect π–A and ΔV–A behaviour: DPPA isotherms move towards more condensed phases with decreasing pH. On the other hand, we found that DPPA isotherms on McIlvaine-EDTA buffer are shifted towards larger molecular areas compared to the water subphase. Previous papers report that surface potential values at maximum packing varies only slightly for pH < 3, whereas beyond this value ΔV was found to increase [21]. Therefore, in the case of McIlvaine-EDTA buffer at pH 4, major variations in ΔV cannot be ascribed solely to a pH effect, but should be correlated to the presence of high concentrations of citrate (6.15×10^{-2} M) in solution [23]. We also recall that McIlvaine-EDTA buffer was found to have a structuring effect at the interface, causing an increase in water surface tension [24]; therefore, a competition between tetracycline and citrate ions at the interface is conceivable. Tetracycline does not affect the behaviour of the ΔV–A isotherms either in the expanded phase or in the condensed one. The above findings indicate that both pH and the ionic strength of the subphase strongly affect the behaviour of DPPA monolayers. In particular, in the presence of McIlvaine-EDTA buffer, the ionic strength of the subphase is so high as to completely mask the contribution of tetracycline at the interface.

3.6. Tetracycline–DPPA interaction mechanism

Further insight into tetracycline–DPPA monolayer interactions can be obtained analysing the difference between the molecular areas of DPPA phospholipid with and without tetracycline in the subphase. The ΔA values as a function of tetracycline concentration are reported in Fig. 7 where we separately describe the expanded phase domain, $\pi = 5$ mN m^{-1} (Fig. 7a), and the condensed one, $\pi = 25$ mN m^{-1} (Fig. 7b).

The behaviour of ΔA as a function of [TC] depends on the pH of the subphase, although at higher surface pressure the variation is considerably smaller. The dashed lines indicate the domains where pH decreases with tetracycline concentration (see also Fig. 3a). In the case of McIlvaine-EDTA buffer, the dotted lines refer to the region where the surface activity of tetracycline cannot be disregarded. For water and TRIS buffer subphases, the concentration range examined falls safely in the domain of constant surface tension $\gamma_{water} = \gamma_{TC}$.

In the case of water subphase, we observe an abrupt increase in surface area up to a threshold concentration, i.e. [TC]* = 1×10^{-4} M both at low and high surface pressure. In the first part of this work [5], we found a similar behaviour for zwitterionic phospholipids, although the threshold concentration occurred in that case at higher [TC]. The presence of a threshold concentration was

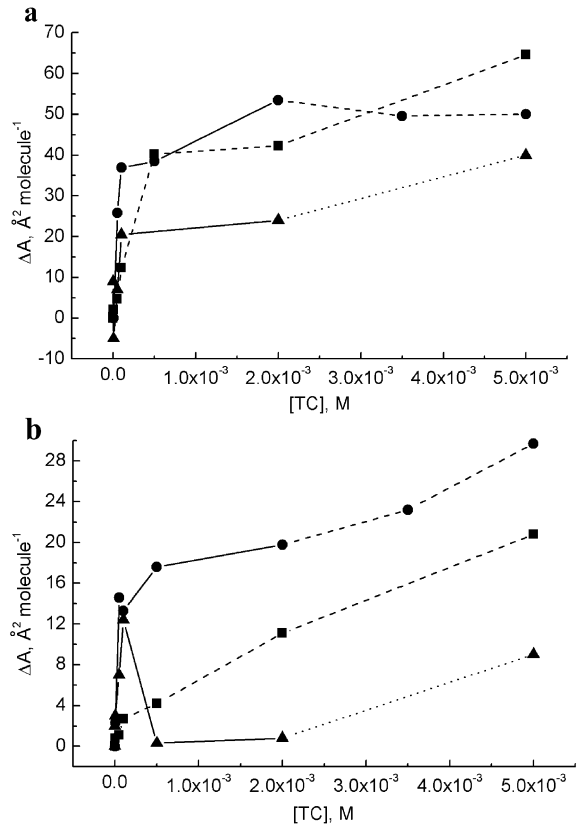

Fig. 7. Difference in surface areas, at constant π, for DPPA monolayers with and without TC in the subphases: TRIS buffer (circles), water (squares), McIlvaine-EDTA buffer (upward triangles). (a) $\pi = 5$ mN m^{-1}; (b) $\pi = 25$ mN m^{-1}.

explained in terms of changes in the distribution of the tetracycline species in solution and hence at the interface: above [TC]* (pH = 4.0) the amount of the neutral H_3TC species decreases with increasing [TC] and the charged species becomes the dominant one in the case of water subphase (see Fig. 3b).

In fact, when we used buffer subphases where the relative concentrations of the different species remain constant, we obtained saturation behaviour for $\Delta A - [TC]$ curves. In the case of TRIS buffer (circles in Fig. 7a), deviations from the saturation behaviour were observed when the pH of the subphase started to decrease, at both high and low surface pressure. Regarding the McIlvaine-EDTA buffer, deviations above $[TC] = 5 \times 10^{-4}$ M should be ascribed to two main factors: the presence of citrate ions at the interface and the high surface activity of tetracycline in McIlvaine-EDTA buffer at these concentrations. Such effects are minor, or negligible, in the liquid-expanded phase at $\pi = 5$ mN m^{-1}.

Similarly, in Fig. 8 we report the behaviour of surface potential as a function of tetracycline concentration in TRIS buffer subphase at constant surface pressure, i.e. $\pi = 5$ mN m^{-1} and $\pi = 25$ mN m^{-1}.

The results for McIlvaine-EDTA buffer subphase are not reported, since the corresponding data are vitiated by the high ionic strength of the buffer solution and by the surface specific interactions of the citrate ions. The data obtained for DPPA on the tetracycline/water subphase were reported and discussed in a previous paper [5]. Analogously to ΔA results, we observed an increase in ΔV as a function of tetracycline concentration up to $[TC] = 10^{-4}$ M; above this concentration ΔV remained nearly constant.

In tetracycline free subphases, the surface potential of an amphiphilic monolayer at the water–air interface can be described [25] as:

$$\Delta V_1 = \Delta V_{\mathrm{DPPA}}^{\mathrm{DIP}} + \Psi_0^1 \tag{3}$$

where the term $\Delta V_{\mathrm{DPPA}}^{\mathrm{DIP}}$ is due to the permanent dipole moment of the phospholipid molecule and Ψ_0, i.e. the double layer potential, is present only in the case of ionized or ionisable monolayers.

The Ψ_0 term in the Eq. (3) was computed using the Gouy–Chapman model [11,22,26], according to which:

$$\Psi_0 = \frac{2kT}{e} \cdot \sinh^{-1}\left(\frac{\sigma}{(8\varepsilon\varepsilon_0 kTc)^{1/2}}\right) \tag{4}$$

where k is Boltzmann's constant, T the absolute temperature, σ the surface charge density equal to $e\alpha/A$ with e as the electronic charge, α the degree of the dissociation of the head group, A the area per molecule, c the ionic concentration of the subphase (M), ε_0 and ε are the vacuum and local dielectric constants, respectively.

For ionisable substances, such as DPPA, the protonation equilibria change with the pH of the subphase and therefore with [TC] in the subphase (see Fig. 3b). DPPA dissociation degree, α, was calculated using literature values for the average interfacial pKs: p$K_1 = 2$ and p$K_2 = 6$ [27] and the results are reported in Fig. 3b. Ψ_0 values, corresponding to the limiting molecular area, were obtained disregarding the $DPPA^{2-}$ species contribution for tetracycline-free subphases.

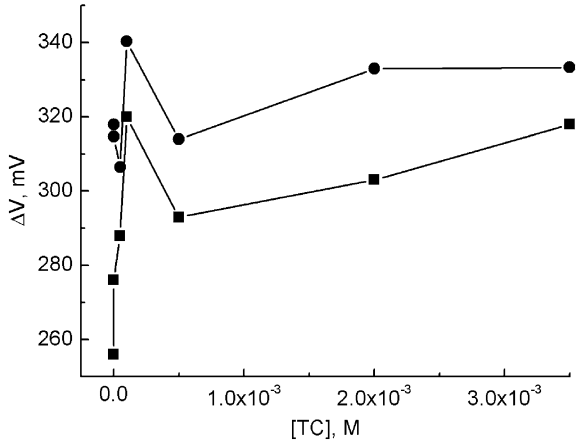

Fig. 8. Surface potential, ΔV, for DPPA monolayers as a function of the tetracycline concentration in the TRIS buffer subphase: $\pi = 5$ mN m^{-1} (squares), $\pi = 25$ mN m^{-1} (circles).

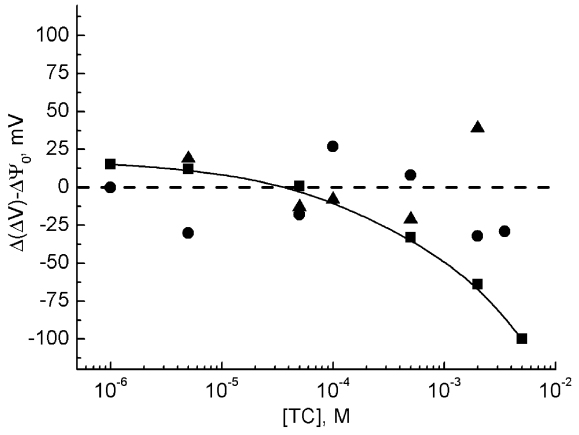

Fig. 9. $\Delta(\Delta V)-\Delta\Psi_0$ as a function of the tetracycline concentration at $\pi=25$ mN m^{-1} for DPPA monolayers: TRIS buffer (circles), water (solid line and squares), McIlvaine-EDTA buffer (upward triangles). The dashed line is an eye guide indicating zero.

In the case of tetracycline-containing subphases, the contributions of both phospholipid and tetracycline at the interface should be considered:

$$\Delta V_2 = \Delta V^{\mathrm{DIP}} + \Psi_0^2 = \Delta V_{\mathrm{DPPA}}^{\mathrm{DIP}} + \Delta V_{\mathrm{TC}}^{\mathrm{DIP}} + \Psi_0^2. \quad (5)$$

The term ΔV^{DIP} is directly correlated to the interfacial density of the dipoles, n, and to the vertical component of the dipole moment, $\mu_\perp$, of the amphiphile [25]:

$$\Delta V^{\mathrm{DIP}} = \frac{n\mu_\perp}{\varepsilon\varepsilon_0} \equiv \frac{n\vec{\mu}\langle\cos\vartheta\rangle}{\varepsilon\varepsilon_0} \quad (6)$$

where $\vec{\mu}$ is the intrinsic dipole moment of the molecule, $\mu_\perp$ is its vertical component and ϑ is the angle between the two vectors. In alternative models [28,29], the term ΔV^{DIP} is further subdivided into the contributions of the polar head group, of the interfacial water layer and of the hydrocarbon tails. Any variation in ΔV^{DIP} could then be ascribed either to a change in the orientation of the DPPA polar groups, to water penetration and/or reorganisation at the interface and, in our specific case, to the effective interfacial contribution of TC, i.e. $\Delta V_{\mathrm{TC}}^{\mathrm{DIP}}$. We computed the quantity $[\Delta(\Delta V)-\Delta\Psi_0]$ from Eq. (7) for the monolayer at maximum packing where the contribution due to the water reorganisation can be disregarded. Assuming that $\Delta V_{\mathrm{DPPA}}^{\mathrm{DIP}}$ is constant, the term $[\Delta(\Delta V)-\Delta\Psi_0]$ should be directly proportional to the dipole contribution of TC to the total surface potential:

$$\Delta(\Delta V) = \Delta V_2 - \Delta V_1 = \Delta V_{\mathrm{TC}}^{\mathrm{DIP}} + (\Psi_0^2 - \Psi_0^1) \quad (7)$$

In Fig. 9, we report the $\Delta(\Delta V)-\Delta\Psi_0=\Delta V_{\mathrm{TC}}^{\mathrm{DIP}}$ values obtained in the condensed region of the isotherms for the three systems.

The data in Fig. 9 show that, in the case of water, $\Delta V_{\mathrm{TC}}^{\mathrm{DIP}}$ is close to zero in the domain of existence of the H_3TC species, whereas it starts to decrease for [TC]$>1\times10^{-4}$ M in correspondence with the appearance of the cationic H_4TC^+ and it reaches $\Delta V_{\mathrm{TC}}^{\mathrm{DIP}}=-100$ mV.

Furthermore, the values of $\Delta V_{\mathrm{TC}}^{\mathrm{DIP}}$ obtained for McIlvaine-EDTA and TRIS buffer are very low and scattered around zero: this may imply either that the tetracycline interfacial density n_{TC} is low or that the vertical component of the dipole moment is almost negligible.

The tetracycline dipole moment was computed with respect to the centre of mass of the molecule, from semi-empirical calculation using either AM1 or PM3 parameterisation. Both methods gave similar results with total dipole moment of 14.0 and 14.8 D for H_4TC^+ and H_3TC, respectively. The dipole moment vector and the electrostatic potential for the H_3TC and H_4TC^+ forms of tetracycline in the folded conformation are reported in Fig. 10a and b, respectively.

The dipole moment vectors, although similar in absolute value, differ in the orientation: the positive end points toward the dimethylamino group in the H_4TC^+ case, whereas in the case of zwitterionic H_3TC the positive end points towards the BCD ring. The orientation of the molecule in the monolayer at maximum packing has been estimated from Eq. (6) using the tetracycline surface density (n_{TC}) calculated as $n_{\mathrm{TC}}=(\Delta A/A_{\mathrm{TC}})\times(A_{\mathrm{DPPA}})^{-1}$ at the limiting area. We estimated A_{TC}, the area requirement for tetracycline at the interface, for two different orientations of the molecule: flat at the interface or with the oxygen-rich part perpendicular to the interface. In the former case, A_{TC} is

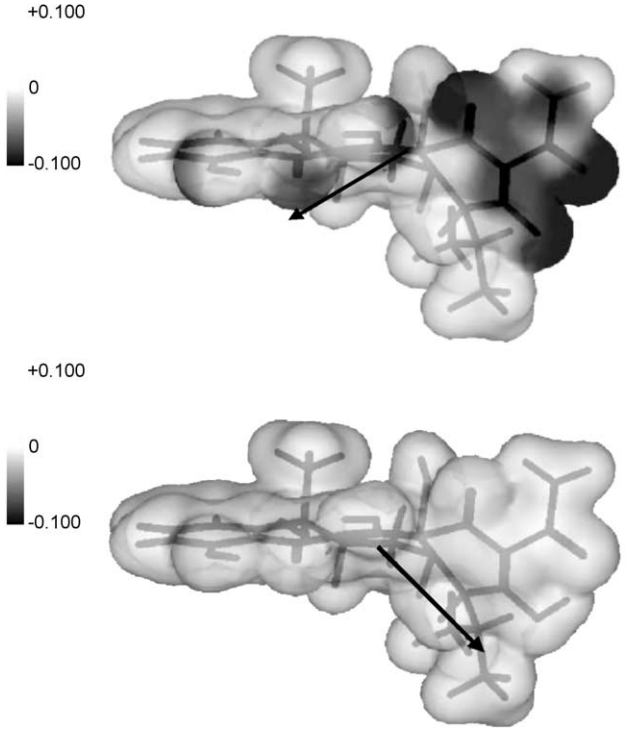

Fig. 10. Electrostatic potential map (the grey scale ranges between -0.1 and 0.1 e/a_0) and dipole moment vector for the H_3TC (a) and H_4TC^+ (b) forms of tetracycline. The vector points towards the positive charge.

100 Å^2 for both the cationic and the zwitterionic forms. In the second case, A_{TC} was found to be slightly smaller, i.e. A_{TC} 80 Å^2. The average angle between the molecule dipole moment and the normal to the interface (θ) is $90 \pm 3°$, for the three systems independently on [TC]. The results obtained for $A_{TC} = 80$ Å^2 produced almost identical θ values and only 20% smaller n_{TC}. In the case of water and TRIS buffer solution at high tetracycline concentrations, the results show that H_4TC^+ is oriented with the BCD rings almost parallel at the interface and the dimethylamino group protruding towards the water phase as shown in Fig. 10b. Such orientation is maintained also upon deprotonation; in fact, θ values always close to 90°, either above or below the water surface, are consistent with the picture reported in Fig. 10a for H_3TC.

The geometrical dimensions of the H_3TC and the H_4TC^+ forms are very similar as it can be seen in Fig. 10a and b. The same cross-sectional area $A_{TC} = 100$ Å^2 was obtained for both forms assuming the flat-like orientation of the molecule (see Fig. 10a and b) with an orientation of the plane of BCD rings parallel at the interface. This choice was also supported by previous results [5] that indicated an angle between μ and the normal to the interface in the range 85–88°.

This information suggests that H_4TC^+ increasingly associates with the DPPA polar groups. Moreover, the negative sign of ΔV_{TC}^{DIP} clearly indicates that μ of H_4TC^+ described in Fig. 10b points away from the air phase, thus exposing the positively charged dimethylamino group deeper into the water phase.

3.7. Models for tetracycline interaction with the monolayer

In order to obtain a more quantitative description of tetracycline/DPPA interactions, we tested two different models for the tetracycline association with the phospholipid monolayer at the interface: either a simple partition model or a specific adsorption of the Langmuir type. The data were analyzed considering partition equilibrium between the tetracycline associated to the monolayer and the tetracycline in the subphase [30].

We define a partition constant K_p as:

$$K_p = X_b/[TC]_i \quad (8)$$

where X_b is given by n_{TC}/n_{DPPA} and $[TC]_i$ is the concentration of tetracycline underneath the monolayer. In the case of charged substances, i.e. H_4TC^+, $[TC]_i$ is defined by the Boltzmann equation as $[TC]_i = [TC]_{eq}\exp(-\Psi_0 F/RT)$ with Ψ_0 interfacial potential. This latter term was found to be low and we also considered $[TC]_i = [TC]_{bulk}$ in the case of the cationic form H_4TC^+, as reported by other authors in similar studies [30]. We computed K_p at different surface pressure and the results for $\pi = 5$ mN m^{-1} and $\pi = 25$ mN m^{-1} are reported in Table 3 for the three subphases. The partition model was found to successfully represent the experimental data only for tetracycline concentrations above the threshold

Table 3
Partition constants (K_p) at $\pi = 5$ mN m^{-1} and $\pi = 25$ mN m^{-1}

[TC], M	$\pi = 5$ mN m^{-1}			$\pi = 25$ mN m^{-1}		
	TRIS buffer	Water	McIlvaine buffer	TRIS buffer	Water	McIlvaine buffer
	K_p, M^{-1}	K_p, M^{-1}	K_p, M^{-1}	K_p, M^{-1}	K_p, M^{-1}	K_p, M^{-1}
5×10^{-6}	>5000	~ 1000	0	4651	~ 190	>5000
5×10^{-5}	5000	~ 1000	1429	2857	~ 190	1429
1×10^{-4}	3333	~ 1000	2000	1250	~ 190	1250
5×10^{-4}	667	~ 1000	–	331	~ 190	–
2×10^{-3}	~ 160	200	100	~ 60	50	0
3.5×10^{-3}	~ 160	140	100	~ 60	50	0
5×10^{-3}	~ 160	80	100	~ 60	50	0

($[TC]^* = 1 \times 10^{-4}$ M) for all subphases. In particular, K_p was found to be almost constant in the case of water subphases at both low and high surface pressures, although its value varies depending on the tetracycline concentration range examined. For $[TC] < [TC]^*$, $K_p = 1000$ M^{-1}, whereas for higher concentration K_p is lower (140 M^{-1}), suggesting lower affinity of TC for the lipid monolayer. The K_p values found for water subphases compare well with the K_p values of 660 and 11 M^{-1} found for the binding of dibucaine and etidocaine to phospholipid monolayers [30]. The results for the McIlvaine-EDTA buffer are similar to the water case at low surface pressure, although more scattered around an average value. At $\pi = 25$ mN m^{-1}, K_p is null suggesting a lack of interactions between the tetracycline and the monolayer. Regarding McIlvaine-EDTA buffer, the results are biased by the competitive presence of the citrate ions. Differently, in the case of TRIS buffer, the partition model fails to describe the data for tetracycline concentrations below [TC]* and better agreement is found for higher concentrations where the low K_p values suggest minor binding for both surface pressures.

The Langmuir adsorption isotherm [30,31] was tentatively used to describe the tetracycline-induced increase in the surface area at constant surface pressure reported in Fig. 7 by means of a least-square fitting procedure:

$$\Delta A = \frac{\Delta A_{sat} \times [TC]}{[TC] + K_d} \quad (9)$$

where ΔA_{sat} is the area occupied by the tetracycline at maximum adsorption and K_d is the dissociation constant. The fit was not always satisfactory; in Fig. 11, we report the fitted and experimental points for TC in the TRIS buffer subphase. The best fits were obtained using only the data points in the region of constant pH, that is to say disregarding tetracycline concentrations above 1×10^{-4} M.

In the case of water, where pH changes over the entire concentration range, the best results were obtained considering only the concentration domain where $[H_4TC^+]$ is unimportant. For McIlvaine-EDTA buffer, the fit was generally poor either because of the interference of citrate ions at the interface and because of the nonnegligible surface tension of tetracycline at high [TC]. The fitting parameters

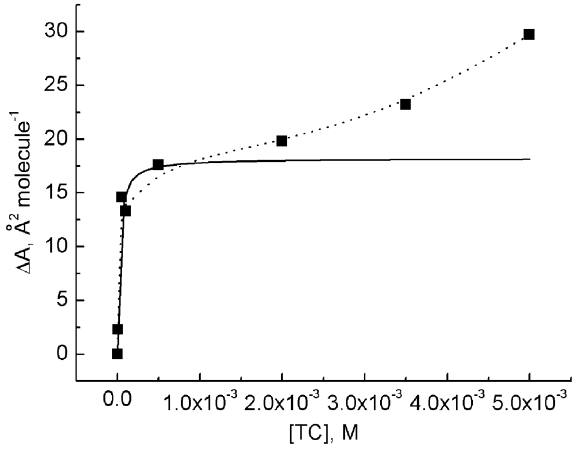

Fig. 11. Langmuir adsorption isotherms for DPPA monolayers $\pi = 25$ mN m^{-1} on tetracycline/TRIS buffer subphase. The dotted line represents the fit over the entire concentration range. The solid line represents the Langmuir fit obtained in the range of constant pH.

obtained for the three systems studied are reported in Table 4. The value of the dissociation constant, K_d, for TRIS buffer is generally lower than water, confirming a stronger binding of the tetracycline to the DPPA monolayer at pH 7. Major differences in the binding affinity between the two surface pressure domains are registered only for McIlvaine-EDTA buffer, whereas for TRIS buffer K_d values are very similar for both surface pressures. The same also holds for water subphases if we consider only the systems where the concentration of the charged species H_4TC^+ is negligible, i.e. $[TC] < 1 \times 10^{-4}$ M. The saturation value, ΔA_{sat}, represents the increment in the DPPA surface area due to the tetracycline at maximum adsorption. ΔA_{sat} can be compared to the area requirement for the folded conformation of tetracycline at the interface ($A_{TC} = 100$ Å^2 as discussed above) providing an estimate of the DPPA/TC molecular ratio at the interface. We obtained 2:1 and 5:1 as molecular ratios for the couple $H_3TC/DPPA^{2-}$ in TRIS buffer at 5 and 25 mN m^{-1}, respectively. At higher [TC] where the couple involved is $H_3TC/HDPPA^-$, the adsorption model fails and the data are successfully fitted with the continuous partition model. For the couple $H_3TC/HDPPA^-$ in McIlvaine-EDTA buffer at pH = 4, we computed 3:1 and 7:1 molecular ratios and the differences are again ascribed to the competitive presence of citrate ions at the interface.

In the case of water, we considered that above pH = 4.0, i.e. $[TC] < 1 \times 10^{-4}$ M, the couple $H_3TC/HDPPA^-$ is the most abundant and the resulting molecular ratio is 1:1 at 5 mN m^{-1} and 2:1 at 25 mN m^{-1}. Below pH = 4.0, the cationic form replaces the zwitterionic tetracycline; the resulting fitting with Eq. (9) is poor in this region, whereas the partition model better describes the $H_4TC^+/HDPPA^-$ interaction.

The comparative analysis of the results allowed to identify the $DPPA^{2-}/H_3TC$ as the most abundant pair in TRIS buffer below [TC]*; in this concentration range tetracycline molecules are adsorbed at the monolayer interface with strong and localized electrostatic interactions between the zwitterionic H_3TC and the $DPPA^{2-}$ groups. Such interactions are adequately described over the entire surface pressure range by a Langmuir adsorption isotherm with a limited number of binding sites and similar dissociation constants.

Above [TC]*, we found that a partition model accounts for the association of the couple $HDPPA^-/H_3TC$ at the interface. This holds at $\pi = 5$ mN m^{-1} and $\pi = 25$ mN m^{-1} although the resulting partition coefficient is higher for low surface pressure.

In the case of water, the couple involved below [TC]* is again $HDPPA^-/H_3TC$ and, similarly to the TRIS buffer system, we found that the partition model better describes the experimental data, especially at high surface pressure. Above [TC]*, that is to say for the $HDPPA^-/H_4TC^+$ pair, the application of both models failed.

Also in the case of McIlvaine-EDTA buffer, the involved couple is $HDPPA^-/H_3TC$ but we found partial agreement with the partition model in the case of very high TC concentration and low surface pressure; anyway, the K_p value is small if compared to the other cases. This partial agreement can be explained taking into account that the competitive association of citrate ions becomes less important at high [TC].

The analysis of the binding/partition data evidences that the interplay of electrostatic interactions dictates the association mechanism of TC with the interfacial DPPA monolayer. Other authors also found similar results for phloretin adsorption at lipid monolayers [32], where strong dipole–dipole interactions between the monolayer and the adsorbed molecule were invoked to account for the binding mechanism.

The rationale for this interpretation is described in Fig. 10: the zwitterionic form H_3TC bears a negative charge diffused on the oxygen rich part of A ring, whereas a positive charge is localized on the dimethylamino group on the A ring for the cationic form, H_4TC^+. On the contrary, the distribution of the electrostatic potential on the BCD rings differs only slightly for the two forms of TC. Changes in the behaviour of the two forms should then be explained

Table 4
Saturation area, A_{sat}, and dissociation constant, K_d, values obtained from the Langmuir fit of the experimental ΔA vs. [TC] curves

Subphase	π, mN m^{-1}	A_{sat}, Å^2 molecule^{-1}	K_d, M	R^2	Species	DPPA/TC
Water	5	105[a]	8×10^{-5}[a]	0.996	$HDPPA^-$	1:1
	25	5	1×10^{-4}[a]	0.953	H_3TC	20:1
TRIS	5	44[b]	3×10^{-5}[b]	0.986	$DPPA^{2-}$	2:1
buffer	25	18[b]	2×10^{-5}[b]	0.974	H_3TC	5:1
McIlvaine-EDTA	5	34	1×10^{-4}	0.819	$HDPPA^-$	3:1
buffer	25	14	3×10^{-5}	0.904	H_3TC	7:1

Predominant pair and DPPA/TC molecular ratio at saturation.
[a] Data fitted only for $[TC] \leq 5 \times 10^{-4}$ M.
[b] Data fitted only in the constant buffer region, $[TC] \leq 1 \times 10^{-4}$ M.

by a specific interactions between the A ring of the molecule and the polar group of the phospholipid. In particular, for the $DPPA^{2-}$ species, the adsorption of the zwitterionic tetracycline results in a strongly localised binding to the monolayer head groups.

4. Conclusions

The results reported and discussed above allow some general conclusions to be drawn on the interaction mechanism of tetracycline with an ionisable phospholipid monolayer at the water–air interface, as well as hypothesising the role of the pH of the subphase in tuning the extent of such interactions.

Although surface activity for the zwitterionic tetracycline was found to be larger than for the charged species, tetracycline generally has a very low tendency to accumulate at the interface in the absence of any amphiphilic monolayer. Migration towards the interface in the presence of phospholipid is dictated by the electrostatics of the system and the interplay of dipole–dipole and ion–dipole interactions. The results collected from the investigation of the spreading isotherms allow us to exclude tetracycline penetration into the lipophilic portion of the monolayer. The data indicate that tetracycline may be incorporated at low surface pressure in the polar region of the monolayer. Upon further compression, tetracycline is pushed deeper down into the water layer underneath the polar head groups, or expelled from the monolayer in the case of McIlvaine-EDTA buffer. The behaviour observed for DPPA monolayers in the presence of tetracycline depends on the type and strength of interactions that can be established between the different species of TC and the phospholipid molecule. For concentrations below 1×10^{-4} M, which is the range of interest for tetracycline in natural systems, the results show that the strength of interaction between the antibiotic and the DPPA monolayer decreases in the TRIS buffer>water> McIlvaine-EDTA buffer series. These findings can be rationalised in terms of different protonated forms of both tetracycline and DPPA. From the comparison of experimental data with semi-empirical calculation, we could sketch a distribution of tetracycline below the polar head group of DPPA as a flat-like orientation with a 2:1 ratio between the phospholipid and tetracycline. This study confirms that such interactions, although strong enough to persist also in the condensed phase, do not seem sufficient to promote penetration of TC through the polar groups up to the hydrophobic layer.

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