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Hexadecylphosphocholine interaction with lipid monolayers

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Abstract

The phospholipid analogue miltefosine or hexadecylphosphocholine (HePC) is a drug of high interest in the treatment for fatal visceral leishmaniasis (VL) due to *Leishmania donovani* particularly because of its activity by oral route. In this study, the interaction of HePC with a monolayer of β-palmitoyl-γ-oleyl-phosphatidylcholine (POPC) as membrane model or sterol (ergosterol or cholesterol) was investigated. At a constant pressure of 25 mN/m, the adsorption kinetics of HePC into the monolayers showed that HePC molecules are inserted into the monolayer of lipids as monomers until the critical micellar concentration (CMC). At HePC concentrations superior to the CMC, the micelles of HePC are deployed at the interface as groups of monomers into the POPC or sterol monolayer. The study of mixture of HePC/(POPC or sterol), spread at the air–water interface, shows that a simple miscibility between HePC and POPC is observed, whereas a high condensation appears between HePC and sterols showing a high affinity between HePC and sterols. In addition, HePC does not act as detergent disturbing membrane integrity.

Keywords: Monolayer; Phospholipid; Sterol; Hexadecylphosphocholine; *Leishmania*

1. Introduction

Leishmaniases are parasitic diseases due to *Leishmania*, a dimorphic protozoan that exists as flagellated mobile promastigotes in sandfly vector and as intracellular amastigote in the mammalian host. Leishmaniases are considered by the World Health Organization to be one of the six major diseases in developing countries [1].

Several species of *Leishmania* are responsible for at least three major forms of infections in man: cutaneous, mucosal and visceral leishmaniasis (VL).

VL infection, particularly due to species of *Leishmania donovani*, is the most damaging form because fatal if untreated. There are annually nearly 0.5 million new cases of VL [2]. Along with Brazil, Bangladesh and Sudan, India contributes to 90% of the global burden of VL [3]. Classic VL presents as fever, hepatosplenomegaly and pancytopenia.

Chemotherapy is the only real means to control this disease since vaccine or vector eradication is still hypothetical. However, chemotherapy has to be improved to overcome the problems of resistance, toxicity and high cost of available drugs. The two pentavalent antimonial compounds, sodium stibogluconate and meglumine antimoniate, administered parenterally as first line chemotherapeutic agents against all forms of leishmaniasis including VL are toxic and widespread resistance is reported mainly in India, Kenya and Sudan [4–7]. In case of antimonial resistance, Amphotericin B (AmB) which binds to membrane sterols [8,9] is used as liposomal formulation (AmBisome®) administered intravenously despite its nephrotoxicity [10–12]. Other lipid formulations of AmB exist and are also administered intravenously [13,14]. The major limitation that prevents more widespread use of these formulations is their high cost. Therefore, it is useful to define low-dose treatment regimens, but they remain too expensive for most endemic countries [15,16].

The phospholipid analogue miltefosine or hexadecylphosphocholine (HePC) (Fig. 1) acquires growing interest

Abbreviations: VL, visceral leishmaniasis; HePC, hexadecylphosphocholine; CMC, critical micellar concentration; POPC, β-palmitoyl-γ-oleyl-phosphatidylcholine

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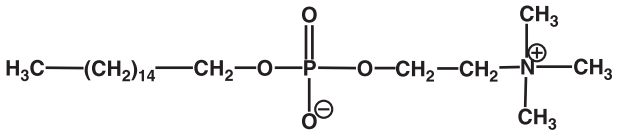

Fig. 1. Chemical structure of HePC.

as a new antileishmanial drug particularly because of its activity by oral route.

HePC was primarily developed as antitumoral agent, particularly for the treatment of cutaneous metastases from breast cancers [17,18]. HePC is clinically efficient in India as oral drug for the treatment of VL. Recent study showed that 94–97% of patients treated orally and daily with miltefosine at approximately 2.5 mg per kilogram of body weight for 28 days were cured and had no relapse after 6 months [19]. Moreover, HePC remains hopeful even for immunodeficient patients with VL [20] and it is also efficient for cutaneous leishmaniasis [21]. However, the mechanism of action of HePC is still unknown.

The uptake of HePC, a water-soluble amphiphilic molecule in eukaryotic cells, may be done by passive diffusion, by the means of a transporter or by endocytosis [22]. We hypothesize that HePC, because of its chemical structure, could have a direct interaction with the cell membrane. In order to assess this assumption, we propose therefore to analyze the physico-chemical interaction between HePC and a phospholipid or a sterol monolayer, a simplified external membrane model.

2. Materials and methods

2.1. Reagents

Miltefosine or hexadecylphosphocholine (HePC) (Fig. 1), an amphiphilic molecule, was kindly provided by Zentaris (Frankfurt, Germany). Solutions of HePC were daily prepared in distilled water (Millipore) or in chloroform/ethanol 1:1 (v/v) at initial concentration of 10^{-3} M. These solutions were immediately used for measurements. Chloroform and ethanol were purchased from Sigma (St. Louis, MO, USA) and were 99% pure and used without further purification.

2.1.1. Lipids

β-Palmitoyl-γ-oleoyl-phosphatidylcholine (POPC, a zwitterionic phospholipid) was chosen for three major reasons: first, because it is an amphiphilic molecule which can spread at the air–water interface and form stable monolayer; second, because of its oleic and palmitic chains and phosphocholine polar head group are major components in biological membranes in eukaryotic cells; third, the mean transition temperature of POPC (Tm = − 3 °C) is close to that of biological membranes phospholipids.

Plasma membranes of eukaryotic cells contain, in addition to phospholipids, varying amounts of sterol. Ergosterol was chosen because it is the major sterol component in addition to cholesterol in *Leishmania* sp. [9,23].

POPC, cholesterol and ergosterol were purchased from Sigma and were 99% pure and used without further purification. For the surface pressure measurements, POPC, cholesterol and ergosterol were dissolved in a chloroform/ethanol 1:1 (v/v) mixture at a concentration of 10^{-3} M.

2.2. Monolayer study

Monolayers were prepared as already described [24] by using a Teflon trough provided by Riegler (Riegler and Kirstein GmbH, Wiesbaden, Germany). The trough (6.2 × 26.3 × 0.5 cm) was filled with NaCl solution (150 mM, pH = 5.6) or pure water (pH = 5.6). The surface pressure was measured with the Wilhelmy method, by means of a thin plate of filter paper. An electronic device enabled us to keep the surface pressure constant by monitoring the displacement of the barriers. This system was used during adsorption experiments. All experiments were performed at 21 ± 1 °C. The speed of compression and decompression of the barriers (3×10^{-2} cm s^{-1}) was kept constant during the experiments.

2.2.1. HePC adsorption at the interface and critical micellar concentration (CMC) determination

HePC was injected at a final concentration in the range from 0.2 to 5 μM into the trough. According to the Wilhelmy plate techniques, we studied the adsorption of HePC at the air–water interface at a constant surface area of 45 cm^2. The variation of surface pressure as a function of time was recorded. The reached maximum surface pressure (P_{max}) was reported as a function of HePC concentrations in order to determine the CMC of HePC.

2.2.2. HePC interaction with lipids (POPC or sterols)

Two techniques were employed to study the POPC/HePC or sterol/HePC interactions.

2.2.2.1. Adsorption of HePC solutions in the presence of a lipid monolayer. A lipid monolayer was obtained by spreading 20 μl of POPC or sterol at initial concentration of 10^{-3} M at the interface. Then, this monomolecular film was compressed until 25 mN/m. Generally, the surface pressure of biological membrane is estimated at 30 mN/m, but reliable measurements must be done at 25 mN/m, pressure not close to the collapse pressure of HePC (Section 3.1.2).

(i) In the first case, the surface pressure (P) was kept constant (25 mN/m) and an aqueous solution of HePC was injected with a microsyringe under the monolayer at a final concentration in the range from 0.2 to 4 μM, according to the process described previously [25]. If an interaction occurred between the molecules of the subphase and the

monolayer, the barriers moved back to keep the pressure to 25 mN/m and the variation of the mean molecular area of lipid versus time was recorded during 60 min (adsorption kinetics). The maximal percentage of HePC monomers (%$HePC_{max}$) inserted into the lipid monolayer is calculated from the following formula [26]

$$\%HePC_{max} = (\Delta A/A_{HePC})/(1 + \Delta A/A_{HePC}) \quad (1)$$

ΔA is the variation of the area per molecule of lipid at the constant surface pressure and A_{HePC}, the cross section of HePC, determined by HePC compression isotherms (Section 3.1.2).

(ii) In the second case, the surface of trough (corresponding to a surface pressure of 25 mN/m) was maintained constant. An aqueous solution of HePC was injected under the monolayer at a final concentration in the range from 0.2 to 4 μM. The variation of surface pressure ΔP of lipid in the presence of HePC was recorded during 60 min.

2.2.2.2. Spreading of mixed lipids: HePC/POPC or sterol. Mixed HePC/lipid organic solutions were prepared in ethanol/chloroform 1:1 (v/v) with various ratios. Twenty microliters of each mixture was spread using a microsyringe at the air–water interface. The mixed films were compressed in order to obtain an isotherm pattern. At a constant surface pressure of 25 mN/m, a phase diagram was obtained by reporting the measured mean molecular area as a function of the HePC molar fraction. The theoretical molecular area obtained if no interaction is assumed between HePC and lipids (additivity curve) was obtained by joining the points corresponding to the molecular area of the lipid alone and the molecular area of HePC alone. If the resulting curve is linear, there is a simple additivity between HePC and the lipid; a concave curve indicates a condensation and, on the contrary, a convex one indicates an extension.

3. Results

3.1. Behavior of HePC alone at the air–water interface

3.1.1. HePC adsorption at the interface and CMC measurements

HePC is composed of two parts, an hydrophobic chain of 16 carbons and a polar head group of PC, which confers it tensioactive properties. This amphiphilic molecule is soluble in water but can form monolayer at the air–water interface. In order to know the state of active HePC against parasites, under the form of monomers or aggregates, we first have to determine the CMC of HePC.

An HePC aqueous solution was injected into the subphase of the Langmuir trough. At low concentrations below the CMC, HePC was essentially soluble in water in the form of monomers. As HePC is an amphiphilic molecule, monomers are able to adsorb at the interface. At concentrations superior to the CMC, there was micelle formation in the trough and saturation of adsorbed monomers at the interface occurred. The maximum surface pressure (P_{max}) was reported as a function of HePC concentrations (Fig. 2) in order to determine the CMC of HePC. The plateau corresponds to the saturation of the surface by the monomers of HePC. The CMC was estimated in the range from 2 to 2.5 μM in the presence of 150 mM NaCl into the subphase and in the range from 2.5 to 3 μM in the presence of distilled water.

3.1.2. HePC compression isotherms

Because of its intermediate solubility, it is possible to dissolve HePC in water as well as in organic solvent. Isotherms of HePC were recorded in three different cases and compared. Either 20 μl HePC dissolved in chloroform/EtOH 1:1 (v/v) or in distilled water at initial concentration of 10^{-3} M was spread at the air–water interface, or 20 μl HePC aqueous solution corresponding to a HePC final concentration of 0.2 μM < CMC was injected in the trough.

The isotherms recorded 60 min after spreading and adsorption of HePC from aqueous solution are represented in Fig. 3 (curves a and b). The obtained low mean molecular area shows that a part of HePC molecules are dissolved in the subphase. On the contrary, the compression isotherms obtained from an organic solution performed 10 min and 1 h after spreading (Fig. 3, curves c and d) are close, indicating that all molecules stay at the interface. Thus, the maximum pressure (collapse) reached in isotherms of HePC was found at 35 mN/m and the minimum molecular area reached was found at 26 $Å^2$. This value is close to the

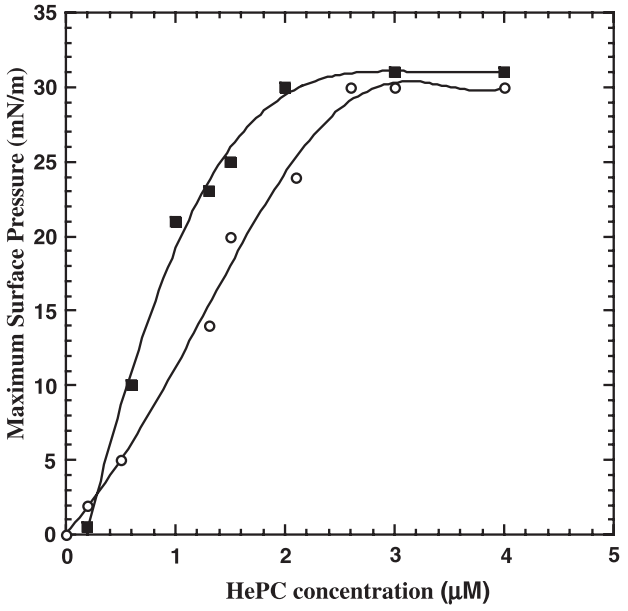

Fig. 2. Determination of HePC CMC: maximal surface pressure obtained from adsorption of HePC at constant area (45 cm^2) versus HePC concentration; (○) distilled water, (■) NaCl concentration: 150 mM. Subphase: pH = 5.6, $T = 21 \pm 1$ °C.

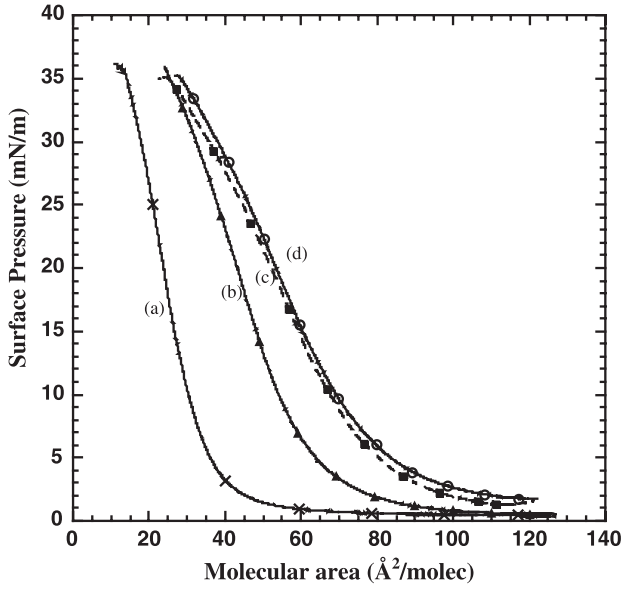

Fig. 3. HePC isotherms recorded: 1 h after injection from an aqueous solution into the trough (curve a), 1 h after spreading from an aqueous solution at the interface (curve b), after spreading from an organic solution at the interface, after 10 min (curve c) and after 1 h (curve d). Subphase: distilled water, pH = 5.6, $T = 21 \pm 1$ °C.

molecular area of a simple carbon chain. Therefore, in further calculations the cross section of the HePC molecule (A_{HePC}) will be estimated at 26 Å^2.

3.2. Interaction of HePC with monolayer of lipid (POPC or sterols)

3.2.1. At constant surface pressure

A lipid monolayer (POPC or sterol) was spread at the interface and compressed until 25 mN/m.

In the first case, this surface pressure (P) was kept constant and an aqueous solution of HePC was injected under the POPC monolayer according to the process described in Section 2.2.2.1. The variation of the mean molecular area (ΔA) of POPC and of ergosterol versus HePC concentration is reported in Fig. 4a and b. The same results are obtained with cholesterol (data not shown). In the two cases, we note that until 2 μM, close to the CMC value (2.5 μM), the area per molecule of POPC increases progressively due to an insertion of HePC monomers into the lipid monolayer. These values are higher in the presence of POPC (Fig. 4a) than sterol (Fig. 4b) because of the state of these lipids: at 21 °C POPC is in liquid expanded (fluid) phase, cholesterol and ergosterol in condensed (ordered) phase. According to the formula (Eq. (1)), at 25 mN/m the maximum percentage of HePC monomers inserted into a POPC monolayer or into a sterol (ergosterol or cholesterol) monolayer, calculated for 2 μM, reached about 28% and 16%, respectively. Above the CMC concentration, curves are not only no linear but values may also considerably vary for one same concentration of HePC. In fact, some micelles of HePC reached interface and were deployed as groups of monomers into the POPC or sterol monolayer, creating an abrupt anarchical increase of the molecular area. The micelles in solution constitute a reserve of monomers for the interface.

3.2.2. At constant molecular area

In the second case, the surface of trough corresponding to a surface pressure of the lipid of 25 mN/m was maintained constant and an aqueous solution of HePC was injected under the monolayer. At this pressure the area per molecule is 69 ± 0.5 Å^2 (Fig. 5) and 34 ± 0.1 Å^2 (Fig. 6), respectively for POPC and ergosterol. Tables 1 and 2 report the variations of surface pressure (ΔP) versus HePC concentration in

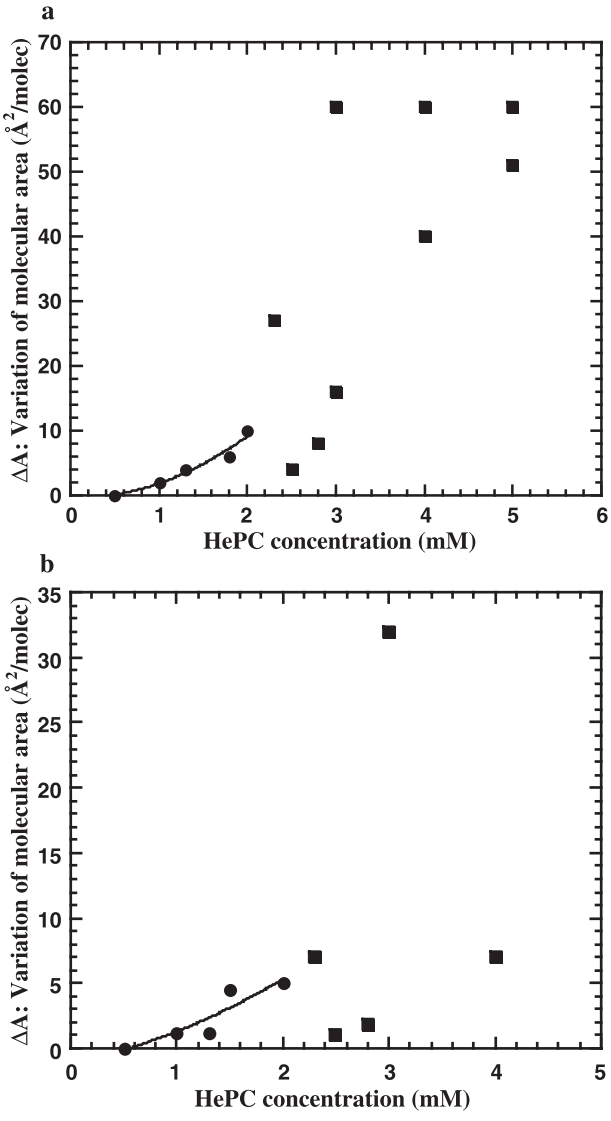

Fig. 4. Variation of the molecular area (ΔA) of POPC (a) and ergosterol (b) as a function of HePC concentration injected at constant surface pressure $P = 25$ mN/m, from an aqueous solution under the monolayer. (●) Monomers; (■) micelles and monomers. Subphase: distilled water, pH = 5.6, $T = 21 \pm 1$ °C.

Table 1
Variations of the surface pressure (ΔP) of POPC after adsorption of HePC from an aqueous solution at constant molecular area of POPC (69 ± 0.5 Å^2)

HePC (μM)	ΔP (± 0.5 mN/m)	
	Instantaneous	After 1 h
0.5	2	2
1	3.5	7
1.5	5	9
2	5.2	10
2.5	8	10
3	8	12
4	8	12

Subphase: distilled water, pH = 5.6. $T = 21 \pm 1$ °C.

the presence of POPC and of ergosterol, respectively, instantaneously and 1 h after injection (the same results are obtained with cholesterol). In the presence of POPC, instantaneous ΔP values increase progressively from 2 to 8 mN/m, whereas in the presence of sterol (ergosterol or cholesterol) these values immediately reach 6 mN/m even at low HePC concentration. On the one hand, as ergosterol and cholesterol are in a condensed state, a very few quantity of adsorbed molecules carries an important variation of pressure. On the other hand, these results can involve a higher affinity of HePC for sterol than for POPC.

One hour after injection and above the CMC of HePC, the maximum variation of surface pressure is 12 mN/m (ΔP_{max}), both in the presence of POPC and sterol corresponding to the saturation of HePC molecules into the monolayer. If we add to this value (12 mN/m) the initial surface pressure of POPC or ergosterol (25 mN/m), we obtain 37 mN/m close to the maximum pressure reached by HePC alone (see Fig. 3 and Section 3.1). Beyond this maximum cohesion pressure, HePC molecules cannot be inserted any more into the lipid monolayer. These results complete those obtained at constant surface pressure: HePC molecules are inserted into the monolayer as monomers until the maximum cohesion of HePC molecules at the interface. The fact that the monolayer pressure did not decrease show that POPC molecules remained at the interface and were not solubilised by HePC micelles. So, HePC does not act as a detergent able to destroy the membrane integrity.

Table 2
Variations of the surface pressure (ΔP) of ergosterol after adsorption of HePC (aqueous solution) at constant molecular area of ergosterol (34 ± 0.5 Å^2)

HePC (μM)	ΔP (± 0.5 mN/m)	
	Instantaneous	After 1 h
0.5	6	6
1	6.5	9
1,5	6	9
2	6.5	10
2,5	6	10
3	6,5	12
4	9,5	12

Subphase: distilled water, pH = 5.6. $T = 21 \pm 1$ °C.

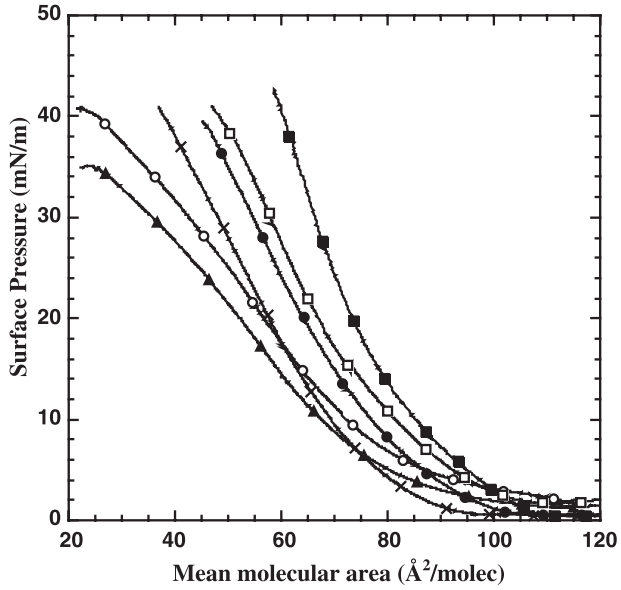

Fig. 5. Isotherm pattern of mixed films of HePC/POPC (mol/mol) at different molar fractions. (▲) HePC, (○) HePC/POPC 80:20, (×) HePC/POPC 40:60, (●) HePC/POPC 30:70, (□) HePC/POPC 20:80, (■) POPC. Subphase: distilled water, pH = 5.6, $T = 21 \pm 1$ °C.

3.3. Spreading of mixed lipids (POPC or sterol/HePC)

To study the relative affinity between lipid (POPC or sterol) and HePC, mixed HePC/lipid organic solutions prepared in ethanol/chloroform 1:1 (v/v) were spread at the air–water interface. The mixed films were compressed in order to obtain an isotherm pattern (Figs. 5 and 6). From

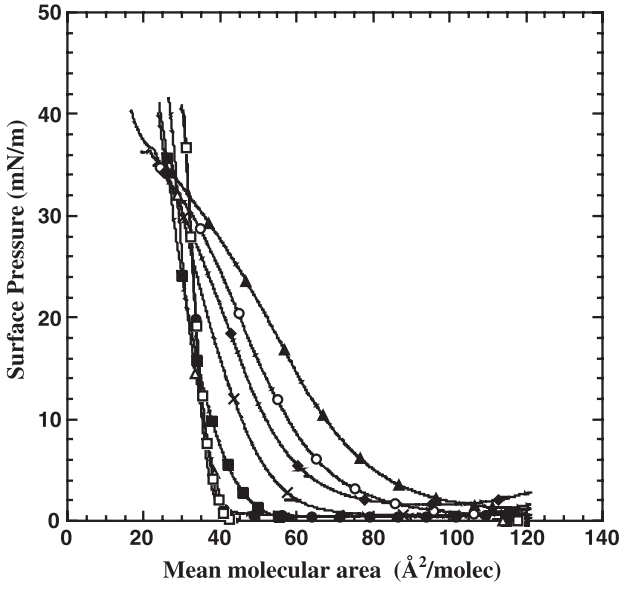

Fig. 6. Isotherm pattern of mixed films of HePC/ergosterol (mol/mol) at different molar fractions. (▲) HePC, (○) HePC/ergosterol 90:10, (♦) HePC/ergosterol 80:20, (×) HePC/ergosterol 60:40 (■) HePC/ergosterol 50:50, (△) HePC/ergosterol 40:60, (●) HePC/ergosterol 20:80, (□) ergosterol. Subphase: distilled water, pH = 5.6. $T = 21 \pm 1$ °C.

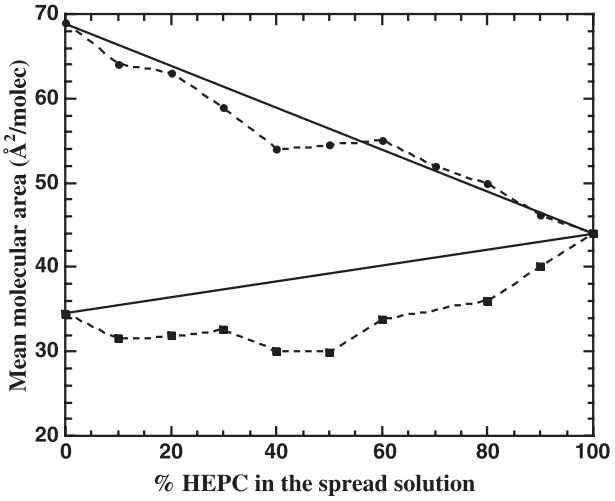

Fig. 7. Phase diagram obtained from isotherms patterns at $P=25$ mN/m. (●) HePC/POPC; (■) HePC/ergosterol. The linear line represents the additivity curve.

these curves a phase diagram was obtained at 25 mN/m (Fig. 7). When we compared the experimental mean molecular area at this pressure obtained for the different mixtures of HePC/POPC (mol/mol) with the theoretical additivity curve (linear curve), we observed that almost all experimental points are close to this line. This means that there is only a simple additivity between POPC and HePC molecules. These results complete those obtained with the adsorption technique (Section 3.2), which underscore a simple insertion of HePC monomers into the POPC monolayer. We can expect also that HePC and POPC are miscible, first, because we do not observe the collapse of HePC, which occurs at 35 mN/m, on the other HePC/POPC isotherms; second, due to the fact that the structures of these molecules are close.

Concerning the mixtures of HePC/ergosterol, we observed that all experimental points are under the additivity curve, indicating an attraction between sterol and HePC. The maximum of condensation is reached at a ratio of HePC/POPC 50:50 (mol/mol). Since sterols are rigid and incompressible molecules, a condensation of HePC with ergosterol occurs, indicating a high affinity between HePC and ergosterol. The same results were obtained with cholesterol (data not shown). This behavior connects with the condensing effect of cholesterol towards phospholipids in general [27–29].

4. Discussion

We have first determined the CMC of HePC to know whether HePC acts as monomers or micelles. The CMC of HePC was estimated at about 2.5 μM in distilled water. This value is fourfold lower than those found by Soares de Araujo et al. [30]. However, this result was obtained under different conditions using 50 mM sodium acetate buffer. The presence of NaCl solution into the subphase has no significant influence on HePC adsorption as we found a similar CMC in distilled water and in 150 mM NaCl [30]. However, our results obtained with lipid monolayers showed that HePC molecules insert into the monolayer as monomers and that some micelles of HePC reaching interface were deployed as groups of monomers into the POPC or sterol monolayer. It can be assumed that HePC insert into the *Leishmania* plasma membrane as monomers and that micelles create a local disorder in the external membrane layer by deployment as monomers. The micelles in solution constitute a reservoir of monomers for the interface. In the research for potential molecular targets of HePC, it was shown that HePC inhibits the specific acyl-CoA acyltransferase enzyme and this inhibition is dose-dependent with an inhibitory concentration of 50 μM up to the CMC of HePC [31]. So we suggest that for its biological activity, HePC must be in concentration above the CMC.

Cell membranes of higher organisms contain in addition to phospholipids varying amounts of cholesterol. The two lipids differ in their swelling properties [32] and their association results in a condensed state [27]. In addition to cholesterol, the major sterol found in the protozoan *Leishmania* plasma membrane is ergosterol [9]. Our biophysical results showed that HePC has a high affinity for sterols and that no selectivity occurs between ergosterol and cholesterol. In addition, we found that HePC, despite its chemical structure, does not act as detergent disturbing the membrane integrity. Finally, we suggest that HePC inserts into the membrane phospholipid by miscibility and interacts with sterols of the membrane.

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