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Interaction of hexadecylbetainate chloride with biological relevant lipids

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Abstract

The present work investigates the interaction of hexadecylbetainate chloride (C_{16}BC), a glycine betaine-based ester with palmitoyl-oleoyl-phosphatidylcholine (POPC), sphingomyelin (SM) and cholesterol (CHOL), three biological relevant lipids present in the outer leaflet of the mammalian plasma membrane. The binding affinity and the mixing behavior between the lipids and C_{16}BC are discussed based on experimental (isothermal titration calorimetry (ITC), Langmuir film balance), and molecular modeling studies.

The results show that the interaction between C_{16}BC and each lipid is thermodynamically favorable and does not affect the integrity of the lipid vesicles. The primary adsorption of C_{16}BC into the lipid film is mainly governed by a hydrophobic effect. Once C_{16}BC is inserted in the lipid film, the polar component of the interaction energy between C_{16}BC and

the lipid becomes predominant. Presence of CHOL increases the affinity of C_{16}BC for membrane. This result can be explained by the optimal matching between C_{16}BC and CHOL within the film rather by a change of membrane fluidity due to the presence of CHOL. The interaction between C_{16}BC and SM is also favorable and gives rise to highly stable monolayers probably due to hydrogen bonds between their hydrophilic groups. The interaction of C_{16}BC with POPC is less favorable but does not destabilize the mixed monolayer from a thermodynamic point of view. Interestingly, for all the monolayers investigated, the exclusion surface pressures are above the presumed lateral pressure of the plasma membranes suggesting that C_{16}BC would be able to penetrate into mammalian plasma membranes *in vivo*. These results may serve as a useful basis in understanding the interaction of C_{16}BC with real membranes.

Keywords: Cationic surfactant, glycine betaine-based ester, membrane model, bilayer, penetration, isothermal titration calorimetry, Langmuir monolayer, computational approach

1. Introduction

Glycine betaine (N,N,N-trimethylglycine) also called betaine is present in various plants, animals and microorganisms. Recently, a review of the synthesis methods and potential applications of glycine betaine derivatives has been published.[1] Betaine can be used to form the hydrophilic part of green surfactants and several surfactants including esters (alkylbetainates chlorides – C_nBC) are based on this molecule.[2-6] These compounds are generally referred to as "mild to the skin"[7] and are used to improve the dermatological properties of other surfactants (e.g., alkylbetaines decrease the skin irritation of anionic compounds). From the point of view of practical applications, the study of the surface properties of these compounds is of great importance. In our previous paper[8], it was found that hexadecylbetainate chloride ($C_{16}BC$) forms a stable monolayer at the air-water interface under all the experimental conditions tested (pH, temperatures, ionic strengths, presence of sodium salts of monovalent or divalent anions).

Surfactants used in personal care products can cause side-effects such as cell membrane damage. Basic studies of surfactant interaction with membranes are thus of great relevance for application purposes, but also for fundamental purposes as membrane-perturbing surfactants are commonly used to lyse cells to study their contents as well as to solubilize their membrane proteins.[9] Considerable research is currently carried out on surfactant-lipid combinations which can be used as drug delivery systems for poorly soluble therapeutic agents.[10] Since C_nBC are a novel class of green surface-active agents potentially useful for the formulation of a wide range of products,[1] in particular for the cosmetic and pharmaceutical fields, a basic physico-chemical study of their interaction with mammalian plasma membrane is required. Due to the complexity of biological membranes, interactions

with major lipid components have been examined individually as a first approach. Bilayers and monolayers of palmitoyl-oleoyl-phosphatidylcholine (POPC), sphingomyelin (SM) and cholesterol (CHOL) have been chosen as simple models. These lipids are representative of the three main types of lipids composing the outer leaflet of the mammalian plasma membrane: phospholipids, shingolipids and CHOL.[11]

In a recent paper[8], we have suggested that primary interaction between C_nBC and lipid monolayers is mainly governed by electrostatic forces. Hydrophobic effect can also play a role when electrostatic interactions cannot occur (in the case of uncharged lipids).

In the current work, the interaction of hexadecylbetainate chloride ($C_{16}BC$) with three types of lipids (POPC, SM, CHOL) in conditions similar to biological ones (pH and ionic strength) is thoroughly examined. The isothermal titration calorimetry (ITC) technique, which measures the heat flow associated with the binding between two entities,[12,13] is used to obtain information about the thermodynamics of $C_{16}BC$ binding to liposomes. Single lipid monolayer models are then used to analyze, by a simple thermodynamic approach, the mixing behavior (miscibility and stability) and the molecular interaction between each lipid and $C_{16}BC$. A computational analysis is also performed in order to gain further insight into the interaction at the molecular level. The results will provide some basic information about the interaction of $C_{16}BC$ surfactant with each of the major components of the mammalian cell membrane individually or with membrane domains that might be highly enriched in a single component.

2. Experimental

2.1. Materials

Hexadecylbetainate chloride (C_{16}BC) (Figure 1) was synthesized in our laboratory[14]. Briefly, betainyl chloride was formed by adding thionyl chloride dropwise to a stirred solution of glycine betaine which was then mixed for ~ 2 hours. C_{16}BC was obtained by the acylation of 1-hexadecanol by betainyl chloride. The molecule's structure was confirmed by Infrared Spectroscopy (IR) and Mass Spectrometry (MS). A typical IR spectrum of betainyl chloride displays bands at 2960, 2870 cm^{-1} (CH_3, C-H stretching), 2925, 2850 cm^{-1} (CH_2, C-H stretching), 1800 cm^{-1} (C=O stretching), 675 cm^{-1} (C-Cl stretching). IR spectra of C_{16}BC displays bands at 2959 cm^{-1} (C-H, stretching), 1751 cm^{-1} (C=O stretching), 1478 cm^{-1} (C-H bending), 1206 cm^{-1} (C-O, stretching). Electrospray ionisation mass spectrum gives the signal of the $[M]^+$ ion at m/z = 342.4. SM, POPC and CHOL (Figure 1) were purchased from Avanti Polar Lipids (Alabaster, AL) (99% purity). The subphase used in this work was a phosphate-buffered saline (PBS) consisting of $NaH_2PO_4.H_2O/Na_2HPO_4$/NaCl 20/20/150 mM adjusted to pH 7.4 with NaOH. The solvents (chloroform and methanol) of analytical grade were obtained from Sigma-Aldrich (St. Louis, MO). Reagents and solvents were used as received.

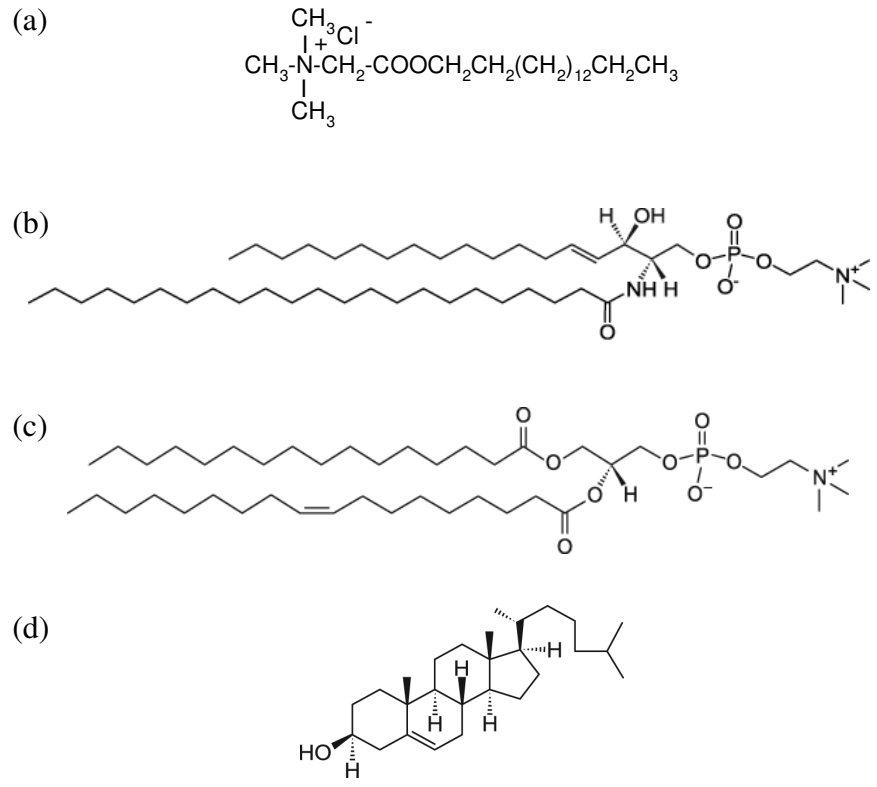

Figure 1: Chemical structure of (a) the hexadecylbetainate chloride (C_{16}BC) and of the three model lipids selected: (b) Sphingomyelin (SM), (c) palmitoyl-oleoyl-phosphatidylcholine (POPC), and (d) cholesterol (CHOL).

2.2. Methods

Preparation of lipid vesicles

Large unilamellar vesicles (LUVs) were used in our experiments to study the interaction of C_{16}BC with lipid bilayers. The vesicles were prepared using the lipid hydration technique. As CHOL is unable to form bilayers[15] and stable LUVs, the interaction of C_{16}BC with this lipid was studied thanks to vesicles prepared with a mixture of SM/CHOL (50:50 mol%). Pure POPC, SM or SM/CHOL was dissolved in chloroform/methanol (2:1 v/v), and introduced into a 10 mL round-bottom flask. A thin lipid film was obtained by removing the

solvents under vacuum with a rotary evaporator. The organic solvent traces were removed by placing the flasks in a dessiccator under vacuum for 16 hours. The dried thin lipid film obtained was hydrated with PBS buffer for 1 h at 30°C for POPC and 60°C for SM and SM/CHOL, and shaken at 10 min intervals. Spontaneously formed multilamellar vesicles (MLVs) were freeze-thawed five times with liquid nitrogen and warm water (30 or 60°C). The dispersion was finally extruded fifteen times through 100-nm polycarbonate filters using a Lipex Biomembranes (Vancouver, BC) extruder to obtain LUVs.

Isothermal Titration Calorimetry (ITC)

ITC measurements were performed by using a VP-ITC (MicroCal, Northampton, MA). All solutions were thoroughly degassed before use by stirring under vacuum or by ultrasonication. The sample cell (1.4565 mL) was loaded with PBS buffer pH 7.4 (blank) or C_{16}BC solution (30 µM) and the reference cell was filled with the PBS buffer. Titration was carried out at 25°C using a 300 µL syringe filled with LUV suspension prepared in PBS pH 7.4. The solution in the sample cell was stirred at 305 rpm during the experiments. A titration experiment consisted of consecutive injections of 5 µL LUV suspension. Each injection took 10 s and a delay of 200 s was applied between each successive injection to allow steady state to be attained. The effective heats were determined by subtracting the values obtained for the blanks from the observed heats. Data were processed using the software provided by the manufacturer (ORIGIN 7 – Originlab, Northampton, USA).

Determination of the thermodynamic parameters of the C_{16}BC binding to the lipid bilayers

The ITC data were treated with the cumulative model described by Heerklotz and Seelig[16] and by Razafindralambo *et al.*[17] according to the following equation:

$$\sum_{k=1}^{i} \delta h_k = \Delta H_D^{w \to b} V_{cell} C_D^0 \frac{K C_L^0}{1 + K C_L^0} \quad \text{Eq 1.}$$

where δh_k is the heat produced following each injection (corresponds to the area of each peak on the heat flow = f(time) plot), $\Delta H_D^{w \to b}$ is the molar enthalpy change corresponding to the transfer of $C_{16}BC$ from the aqueous phase (w) to the bilayer membrane (b), V_{cell} is the volume of the calorimeter sample cell (1.4565 ml), C_D^0 and C_L^0 are the concentrations of $C_{16}BC$ and of lipid in the calorimeter sample cell, respectively, and K is the binding constant.

K and $\Delta H_D^{w \to b}$ can be evaluated simultaneously by a fit of the measured cumulative heat as a function of C_L^0.

The corresponding free energy $\Delta G_D^{w \to b}$ and the reaction entropy $\Delta S_D^{w \to b}$ were then calculated by the standard equations:

$$\Delta G_D^{w \to b} = -RT \ln(K C_w) = \Delta H_D^{w \to b} - T \Delta S_D^{w \to b} \quad \text{Eq 2}$$

with R = 8.31 J.mol^{-1}.K^{-1} and C_w=55.5 M.[16]

Size measurements of lipid vesicles

Size of lipid vesicles prior to and after ITC experiments consisting of titration of $C_{16}BC$ solution (30 µM) by lipid vesicles were determined at 25°C by the dynamic light scattering technique (Delsa Nano C-Beckmann Coulter - 30 mW He-Ne laser, λ =658 nm). Fluctuation of light scattering was measured at an angle of 160 degrees. The Non-negative least square

(NNLS) method included in the software of the instrument was used to calculate the hydrodynamic diameter of the particles. Each value reported is the average of at least three independent measurements.

Langmuir trough technique

Penetration experiments of C_{16}BC into a lipid monolayer were performed at constant area (120 cm²) using an automated LB system (KSV Minitrough, KSV Instruments, Helsinki, Finland). A platinum Wilhelmy plate was used to measure the surface pressure. The PBS subphase temperature was maintained constant at 25 ± 1°C by circulating water through the base plate on which the trough is mounted. The system was enclosed in a Plexiglas box in order to minimize water evaporation and to avoid trace pollution. C_{16}BC stock solutions were prepared in PBS. Lipid monolayers were prepared at defined initial surface pressures (Π_i = 5, 10, 20 and 30 mN/m) by carefully spreading pure lipid solutions (SM, POPC or CHOL) prepared in chloroform/methanol (2:1, v/v) onto the PBS subphase (volume ~ 89 mL). After waiting for stabilization of Π_i, C_{16}BC was injected into the subphase to a final concentration of 1.18 µM. It is assumed that C_{16}BC molecules exist mainly as monomers within the subphase as this concentration is well below the C_{16}BC's measured critical micellar concentration (34 ± 3 µM) (data not shown). During the measurements the subphase is continuously gently stirred with a magnetic bar. The difference between $\Delta\Pi$ of two independent sets of measurements was less than 0.5 mN/m.

Surface pressure (Π)–molecular area (A) compression isotherms were recorded using the same LB system. C_{16}BC and lipids (SM, POPC and CHOL) were dissolved in chloroform/methanol (2:1, v/v). Pure solutions as well as binary mixtures (C_{16}BC/SM, C_{16}BC/POPC and C_{16}BC/CHOL with a defined composition) were prepared to a final concentration of 1500 µM. A volume of 20-30 µL was spread on the PBS subphase at 25°C.

After waiting for 15 min to allow for solvent evaporation and spreading of the molecules, the monolayer was compressed by two barriers approaching symmetrically at a rate of 10 mm/min. The surface pressure was measured during the entire compression. The variation coefficient of at least two independent experiments did not exceed 5%.

Molecular modeling of C_{16}BC/lipid interaction

The organization and mode of interaction of the C_{16}BC and the lipids were studied in C_{16}BC/POPC, C_{16}BC/SM and C_{16}BC/CHOL 1/1 molar complexes.

The modeling approach (called hypermatrix), published more than 15 years ago[18] and upgraded[19], is a "static" approach in the sense that the structure of the molecules is rigid. This method allows to calculate the interaction between a molecule (pharmacological drug, peptide, lipid analog,…) and lipids. Both molecules are first oriented at the lipid/water interface using TAMMO procedure.[20] This allows taking implicitly the hydrophobicity of the medium into account. Then, the molecule of interest (here C_{16}BC) is fixed at the interface and the molecule of lipid (POPC, SM or CHOL) is moved toward C_{16}BC, taking the position of the interface into account. By rotations and translations, more than 100.000 positions are tested, and the corresponding interaction energy is calculated for each position. The energy is the sum of the electrostatic, Van der Waals and hydrophobic contributions. The position corresponding to the lowest energy state of the complex is retained. It should be noted that the calculations are not made in a free space, since the molecules are oriented at the interface (and so the variation of the dielectric constant is notably taken into account) and due to the fact that we calculate the hydrophobic energy using an empirical equation which has been validated by comparison to experiment.[21] This approach implicitly takes the medium into account. This method has been used for studying the interaction of numerous

molecules with lipids[22,23] All calculations were performed on a Linux station bi-xeon quad core, using Z-ultim software.

3. Results

3.1. Thermodynamic analysis of the binding of $C_{16}BC$ to lipid bilayers

A typical isothermal titration of a $C_{16}BC$ aqueous solution with a LUV suspension is shown in Figure 2a. The binding of $C_{16}BC$ to the lipid bilayer is exothermic. The area of the peak decreases gradually with the successive LUV injections and becomes small and constant once free $C_{16}BC$ molecules are no longer available. The residual heat flows observed in the raw data correspond to the heat associated with the vesicle dilution in the sample cell.[24] Integration of the heat flow peaks in Fig. 2a gives the heats of reaction, δhi. Figure 2b shows the cumulative heats ($\sum\delta hi$) as a function of the lipid concentration (C^0_L) in the cell for the three lipids selected.

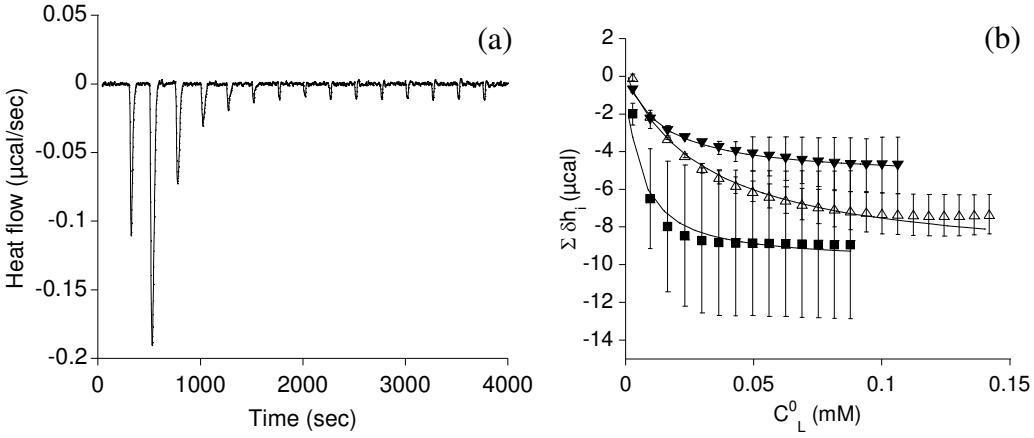

Figure 2: (a) Typical raw data of an ITC experiment. The peaks are related to the successive injections of 5µl of SM/CHOL LUV suspension at 1000 µM into a 30 µM solution of $C_{16}BC$ at 25°C. (b) The cumulative heats of binding ($\sum\delta hi$) as a function of the lipid concentration in the cell (C^0_L). (■) SM/CHOL, (Δ) SM, (▼) POPC. The solid line represents the best fit using the Eq 1. The buffer used was PBS at pH 7.4. Error bars are based on reproducibility over at least two independent measurements.

The integrity of the lipid vesicles after $C_{16}BC$ binding was checked by particle size measurements before and after ITC experiments. The mean hydrodynamic diameter of the vesicles and the size distribution are not greatly affected by the presence of $C_{16}BC$ at 30 μM (Fig. 3). The macroscopic structure of the vesicles is thus not modified by their interaction with $C_{16}BC$. No population of small particles is observed suggesting that no mixed micelles are formed at this concentration of $C_{16}BC$.

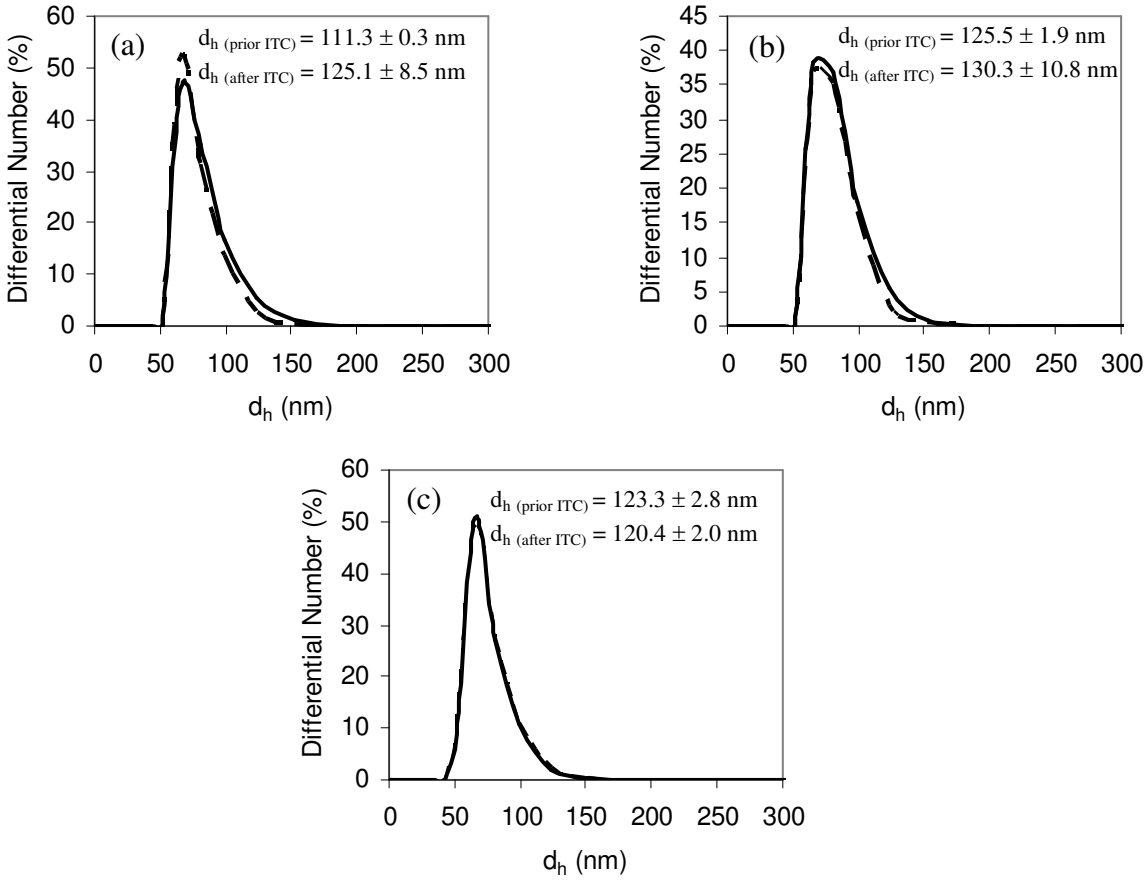

Figure 3: Particle size distribution prior to (full line) and after (dashed line) ITC experiments (a) POPC, (b) SM, (c) SM/CHOL. Inset: value of the mean hydrodynamic diameter (d_h) (average of three independent measurements).

Fitting of the data of Figure 2b according to Eq. 1 and 2 gives the thermodynamic parameters listed in Table 1. It was assumed as for cetylpyridinium chloride (CPC), another positively charged ammonium detergent of similar size to $C_{16}BC$,[25] that the $C_{16}BC$ flip-flop rate from the external to the internal sheet of the bilayer is fast enough to consider, in Eq. 1, the total and not only half of the lipid concentration.

Table 1: Thermodynamic parameters for the binding of $C_{16}BC$ to LUV with different compositions at 25°C.

Bilayer composition	K (mM^{-1})	$\Delta H_D^{w\to b}$ (kJ mol^{-1})	$T\Delta S_D^{w\to b}$ (kJ mol^{-1})	$\Delta G_D^{w\to b}$ (kJ mol^{-1})
POPC	35.3 ± 2.3	-0.72 ± 0.19	35.17 ± 2.52	-35.90 ± 2.34
SM	41.6 ± 11.0	-0.88 ± 0.18	35.32 ± 0.92	-36.20 ± 0.73
SM/CHOL	195.2 ± 13.6	-0.93 ± 0.41	39.20 ± 0.58	-40.13 ± 0.17

Regardless the composition of the vesicle, the binding reactions are spontaneous ($\Delta G_D^{w\to b} < 0$), exothermic ($\Delta H_D^{w\to b} < 0$), and generate a large positive change of the system entropy ($\Delta S_D^{w\to b} > 0$) suggesting that the global binding is mainly entropy-driven. Tsao *et al.*[26] have also concluded for CPC that its insertion into bilayers is driven by an entropy gain. Marcotte *et al.*[20] have suggested that this entropy gain is related to the hydrophobic effect, i.e. a release of water molecules from the hydration layer of the lipid membrane and the dehydration of the $C_{16}B^+$ ion upon binding.[27,28,29]

The binding constant, K, is not significantly different for the pure POPC or SM vesicles. For the SM/CHOL, K is much higher than for the pure POPC or SM vesicles. This indicates that

C_{16}BC has a higher affinity for the SM/CHOL bilayer and that CHOL favors the binding of C_{16}BC to lipid vesicles. It is an opposite behaviour to the one reported for CPC for which CHOL-containing membranes restrict its insertion.[27] The favorable insertion of C_{16}BC into SM/CHOL system could be accompanied by a higher release of water molecules which could explain the larger entropy gain ($\Delta S_D^{w\to b}$) observed in this case.

At 25°C, the binary mixture SM/CHOL (50:50) is in a liquid-ordered state[30,31] which is an intermediate situation between the solid-ordered and the liquid-disordered phases formed by SM and POPC respectively. A uniform lateral distribution of SM and CHOL without domain coexistence has been suggested.[32] Hence, the fluidity of the membrane does not appear to be a critical parameter for C_{16}BC insertion, which is in contradiction with the general belief that a fluid phase is required for the incorporation of biomolecules into bilayers. We suggest that distinct interactions of C_{16}BC with the individual lipids could be at the origin of the different affinities observed rather than a change of membrane fluidity.

To further investigate our assumption, interaction of C_{16}BC with pure lipid monolayers of single lipid was explored using the Langmuir trough technique.

3.2. C_{16}BC penetration into pure lipid monolayers

The ability of C_{16}BC to interact with each lipid is studied by measuring the C_{16}BC-induced surface pressure increase ($\Delta\Pi$) after the injection of C_{16}BC beneath the pure lipid monolayers preformed at different initial surface pressures (Π_i) (5, 10, 20 and 30 mN/m).

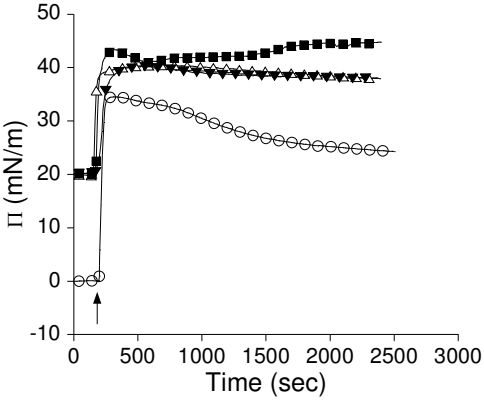

Figure 4: Typical adsorption kinetic of hexadecylbetainate chloride (C_{16}BC) at a clean air-water interface (o) or into lipid monolayers spread at 20 mN/m: (■) CHOL, (Δ) SM, (▼) POPC. The arrow indicates the moment at which injection occurred. C_{16}BC is injected at a final concentration of 1.18 µM into the subphase (PBS pH 7.4; 25°C).

The adsorption of C_{16}BC at a clean air-water interface is instantaneous. Π attains a maximal value (~ 34.5 mN/m) and progressively decreases to reach an equilibrium (~ 24.0 mN/m) after 2000 sec (Fig. 4). The adsorption of C_{16}BC into the lipid interfaces is also instantaneous. The ΔΠ observed gives evidence of penetration of C_{16}BC into the lipid monolayer as explained by Marsh[33] and reported for other surfactants.[34,35] The first step of the kinetics is similar for all the lipids investigated. Π attains a maximal value and progressively decreases to reach the equilibrium for the SM and POPC monolayers. However, in the case of CHOL, Π decreases abruptly after its steep increase and then progressively rises again to reach equilibrium after ~ 40 min. After its initial adsorption at

the CHOL interface, a multi-step molecular reorganization of the monolayer components likely occurs within the monolayer in order to optimize the interface arrangement.

Fig. 5 shows the $\Delta\Pi$ induced by $C_{16}BC$ adsorption into the lipid monolayer as a function of Π_i. $\Delta\Pi$ globally decreases with Π_i as it is generally observed for surfactants.[35] In the case of SM and CHOL, a first order decrease is observed while two parts can be distinguished for the POPC curve. In this latter case, $\Delta\Pi$ decreases slightly with Π_i below 10 mN/m, while it decreases more steeply above 10 mN/m.

By extrapolating the curve of $\Delta\Pi$ versus Π_i to the X-axis, the exclusion surface pressure of penetration (Π_e) can be determined. It corresponds to the initial surface pressure above which the surfactant can no longer penetrate the monolayer. It reflects the penetration power of a surfactant. The Π_e are 34.2, 45.7 and 48.9 mN/m for POPC, CHOL and SM, respectively. They are above the presumed lateral pressure of the biological membranes which is estimated to be around 30 mN/m.[33] Molecules with Π_e higher than 30 mN/m are considered as potentially efficient for interaction and insertion into lipid membranes *in vivo*.[36]

Moreover, the ordinates at the origin of the linear regression for the three systems investigated (30.2 , 40.8 and 31.9 mN/m for POPC, CHOL and SM respectively) are much higher than the equilibrium $\Delta\Pi$ (~ 24.0 mN/m - Fig. 4) obtained after the adsorption of $C_{16}BC$ at the air-water (PBS, pH 7.4) interface in the absence of lipid monolayer. In PBS buffer, all the lipids tested exert thus a positive effect on $C_{16}BC$ adsorption. In a pure water medium, it was the case for CHOL but not for POPC.[14]

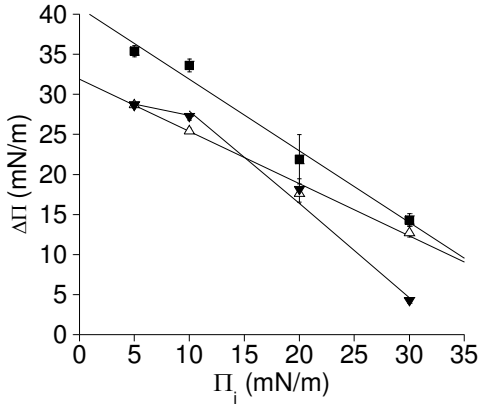

Figure 5: Surface pressure increase ($\Delta\Pi$) caused by the penetration of $C_{16}BC$ into pure monolayers of (■) CHOL, (Δ) SM, (▼) POPC as a function of their initial surface pressure (Π_i). $C_{16}BC$ is injected beneath the lipid monolayer at a final concentration of 1.18 μM in the PBS subphase at pH 7.4 and 25°C. Error bars are smaller than symbols in some cases. The solid line represents the linear fitting of the data (R^2 = 0.98 and 0.99 for CHOL and SM respectively. For POPC, the linear fitting has been done for $\Pi_i \geq 10$ mN/m and R^2 = 0.98).

3.3. Interfacial properties of spread $C_{16}BC$/lipid monolayers upon compression

Binary mixtures of $C_{16}BC$ and each characteristic lipid chosen were spread at the air-water (PBS pH 7.4) interface at 25°C and the compression isotherms of the mixed monolayers were studied. A thermodynamic analysis is performed in order to characterize the interaction between this surfactant and the lipids.

Fig. 6a-c illustrates the Π-A isotherms for pure monolayers of $C_{16}BC$, POPC, SM and CHOL, as well as for the binary mixtures of $C_{16}BC$ with the three lipids in different proportions. The shape of the compression isotherm recorded for $C_{16}BC$ is typical of a condensed monolayer (Fig. 6a). The molecular area (A_c) and the surface pressure (Π_c) at the collapse are 19.4 ± 0.4 Å²/molecule and 46.2 ± 0.5 mN/m, respectively. A_c is higher and Π_c

lower with the PBS subphase than with the pure water subphase used in our previous study (17.3 ± 0.3 Å²/molecule and 52.3 ± 0.6 mN/m).[14] This is the result of the interaction between the positive head groups of C_{16}BC molecules and the subphase hydrophilic anions ($H_2PO_4^-$, $HPO_4^{2-}$and Cl^-) which expands and slightly destabilizes the C_{16}BC monolayer.[37]

The compression isotherm recorded for POPC (Fig. 6a) is characteristic of a liquid expanded monolayer with no discontinuities suggestive of phase changes, in accordance with the literature.[38] The collapse parameters are A_c = 58.5 ± 0.9 Å²/molecule and Π_c = 40.8 ± 1.7 mN/m. The isotherm recorded for SM is also typical of liquid expanded monolayers until ~25 mN/m at which a slight increase in slope is observed suggesting a more condensed state for the monolayer (Fig. 6b and Supporting Information). The shape is similar to those of the isotherms previously obtained.[39] The A_c is 43.1 ± 1.5 Å²/molecule and Π_c is 43.7 ± 2.1 mN/m. The shape of the compression isotherm for CHOL (Fig. 6c) indicates that this sterol forms a highly condensed monolayer at the air–water interface. An area of 40.6 A²/molecule at 20 mN/m is in agreement with the literature.[40,41,42] The collapse parameters observed are A_c = 34.1 ± 0.8 Å²/molecule and Π_c = 43.2 ± 0.1 mN/m.

The compression isotherms for mixed systems (C_{16}BC/POPC, C_{16}BC/SM and C_{16}BC/CHOL) lie between those of the pure components. Increase of the C_{16}BC concentration shifts the isotherms towards the curve of pure C_{16}BC. The shape of the mixed isotherms is not significantly influenced by the proportion of C_{16}BC. Their Π_c values are between those of the pure components with the exception of the mixed C_{16}BC/SM monolayers for which Π_c is much higher for all the proportions studied.

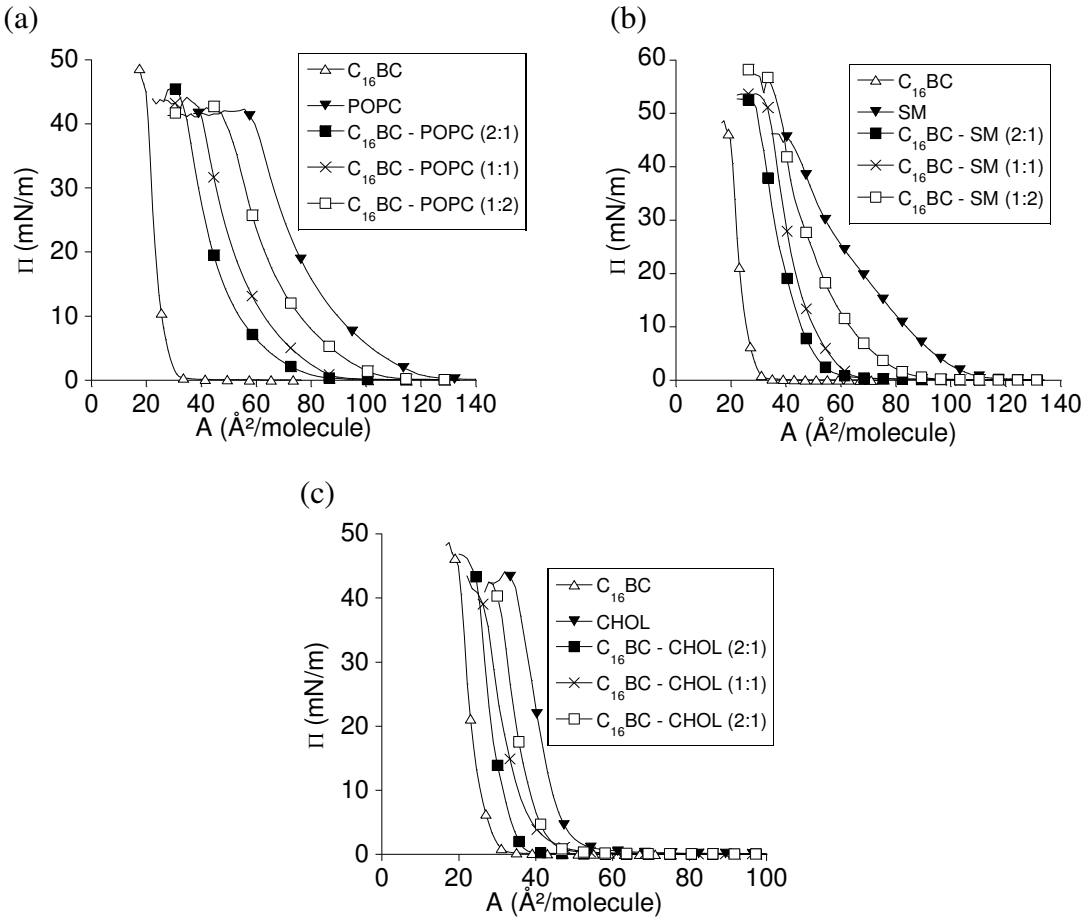

Figure 6: Surface pressure (Π)–molecular area (*A*) isotherms for C_{16}BC and lipid mixtures spread onto PBS subphase pH 7.4 at 25°C: C_{16}BC/POPC (a), C_{16}BC/SM (b) and C_{16}BC/CHOL (c).

A simple thermodynamic analysis of compression isotherm data can give further information about the miscibility and the interaction between the components in the mixed monolayers. The plots of the mean molecular area (A) of the mixed monolayer as a function of the C_{16}BC molar ratio are shown in Fig. 7 for three Π (10, 20 and 30 mN/m). If the components of the monolayer are completely immiscible or ideally miscible, the molecular area is a linear function of the composition and follows the additivity rule given by equation

3. A deviation from ideal behavior suggests the existence of interaction between the molecules and a partial miscibility.[39]

$$A_{12} = A_1 X_1 + A_2 X_2 \qquad \text{Eq. 3}$$

where A_{12} is the mean molecular area for ideal mixing of the two components at a given Π, A_1 and A_2 are the molecular areas of the respective components in their pure monolayers at the same Π and X_1 and X_2 are the molar ratios of components 1 and 2 in the mixed monolayers.

A small positive deviation from ideal behavior is observed for most of the C_{16}BC/POPC monolayers at $\Pi \leq 20$ mN/m. In the case of SM, a negative deviation of A is observed for C_{16}BC molar ratio of 0.5 at 10 and 20 mN/m while no or small positive deviations are noted at 30 mN/m. For these monolayers, interaction occurs between the components whereas an ideal behavior is observed for the C_{16}BC/CHOL for the whole range of compositions and Π investigated. The mean molecular areas of C_{16}BC and CHOL are not influenced by the presence of the other. In other words, it indicates that no interaction exists between the components. In such a case, there are two possibilities for their distribution within the monolayer: either the two components are completely immiscible or they are completely miscible.

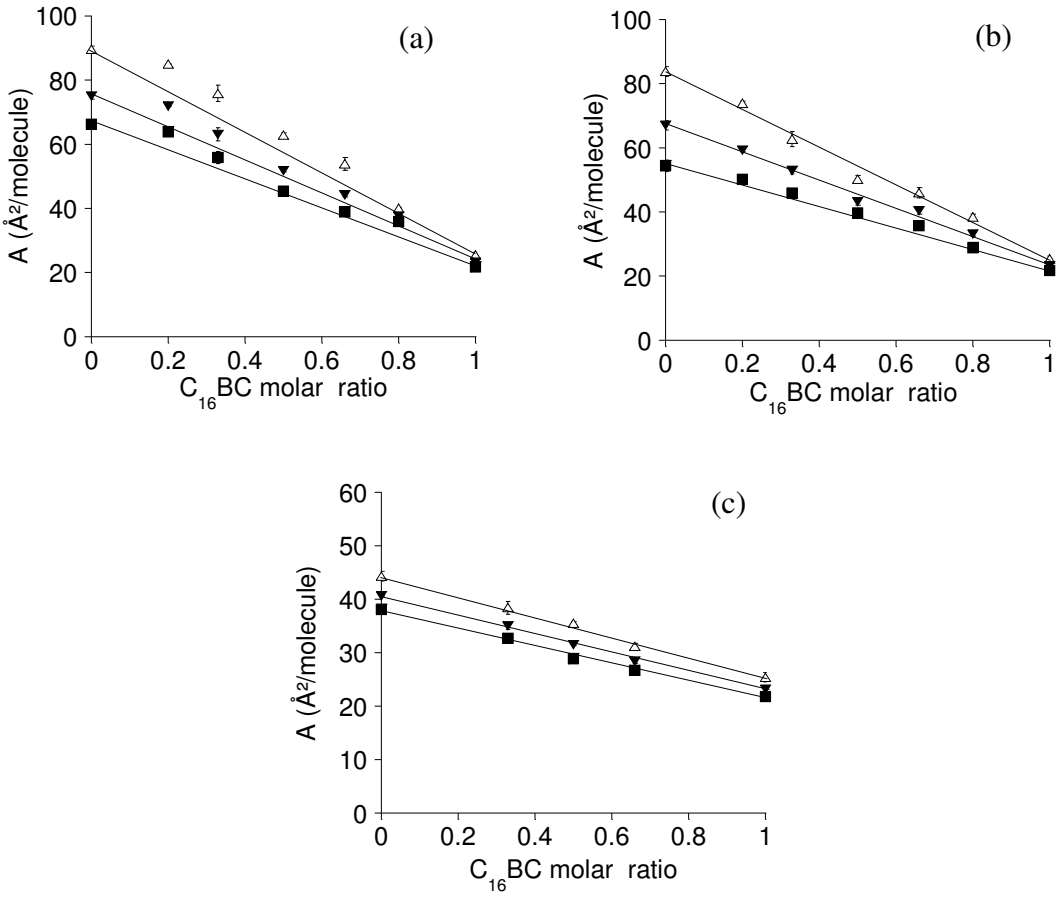

Figure 7: Mean molecular area (A) versus composition plot for the mixtures of C_{16}BC with POPC (a), SM (b) and CHOL (c) at different Π ((Δ) 10 mN/m, (▼) 20 mN/m) and (■) 30 mN/m) at 25°C. Straight lines correspond to the ideal mixing behavior (Eq. 3). In some cases, the error bars are smaller than the symbols.

A more quantitative thermodynamic analysis of the mixtures can be obtained by calculating the excess free energy of mixing ΔG^{ex} (eq. 4) and the total free energy of mixing (ΔG^{M}) (eq. 5).[43]

$$\Delta G^{ex} = \int_0^{\Pi} (A_{12} - X_1 A_1 - X_2 A_2) d\Pi \qquad \text{Eq. 4}$$

$$\Delta G^{M} = \Delta G^{ex} + \Delta G^{id} \qquad \text{Eq. 5}$$

where ΔG^{id} is the free energy for ideal mixing and is defined by equation 6.

$$\Delta G^{id} = RT(X_1 \ln X_1 + X_2 \ln X_2) \quad \text{Eq. 6}$$

where R is the universal gas constant and T, the absolute temperature.

ΔG^{ex} provides information about the possible interaction between the monolayer forming components.[34]

Negative values of ΔG^{ex} indicate that the interactions between the components in the mixed monolayers are more attractive or less repulsive compared to those occurring in their respective pure monolayers. Positive values of ΔG^{ex} suggest that the interactions are less attractive or more repulsive than those existing in the one-component monolayers.[44,45,46]

ΔG^{M} gives information about the thermodynamic stability of the mixed monolayers. A negative value indicates that the system is stable.[34]

ΔG^{ex} and ΔG^{M} as a function of the C_{16}BC molar ratio plots are shown in Fig. 8a-f. Globally, their absolute values are lower than those observed for similar systems such as dialkyldimethylammonium bromides-sterol[44]. Nevertheless, they are statistically different from ideal mixing and provide some further insight into the different affinities between C_{16}BC and the three bilayer components. The positive values of ΔG^{ex} obtained for most of C_{16}BC/POPC mixed monolayers (Fig. 8a) indicate repulsive interaction between the monolayer forming components. This suggests that, at least one component forms two-dimensional aggregates at the interface.[34,46] C_{16}BC/SM mixed monolayers show nil or negative ΔG^{ex} at C_{16}BC molar ratio ≤ 0.5 (Fig. 8b). This suggests that attractive interaction exists within the mixed monolayers between C_{16}BC and SM molecules. However, positive deviations, that can be related to repulsive interactions, are observed at C_{16}BC molar ratio = 0.66 and 0.8. No deviation is observed for the C_{16}BC/CHOL pairs (Fig. 8c) confirming the ideal behaviour of these mixtures.

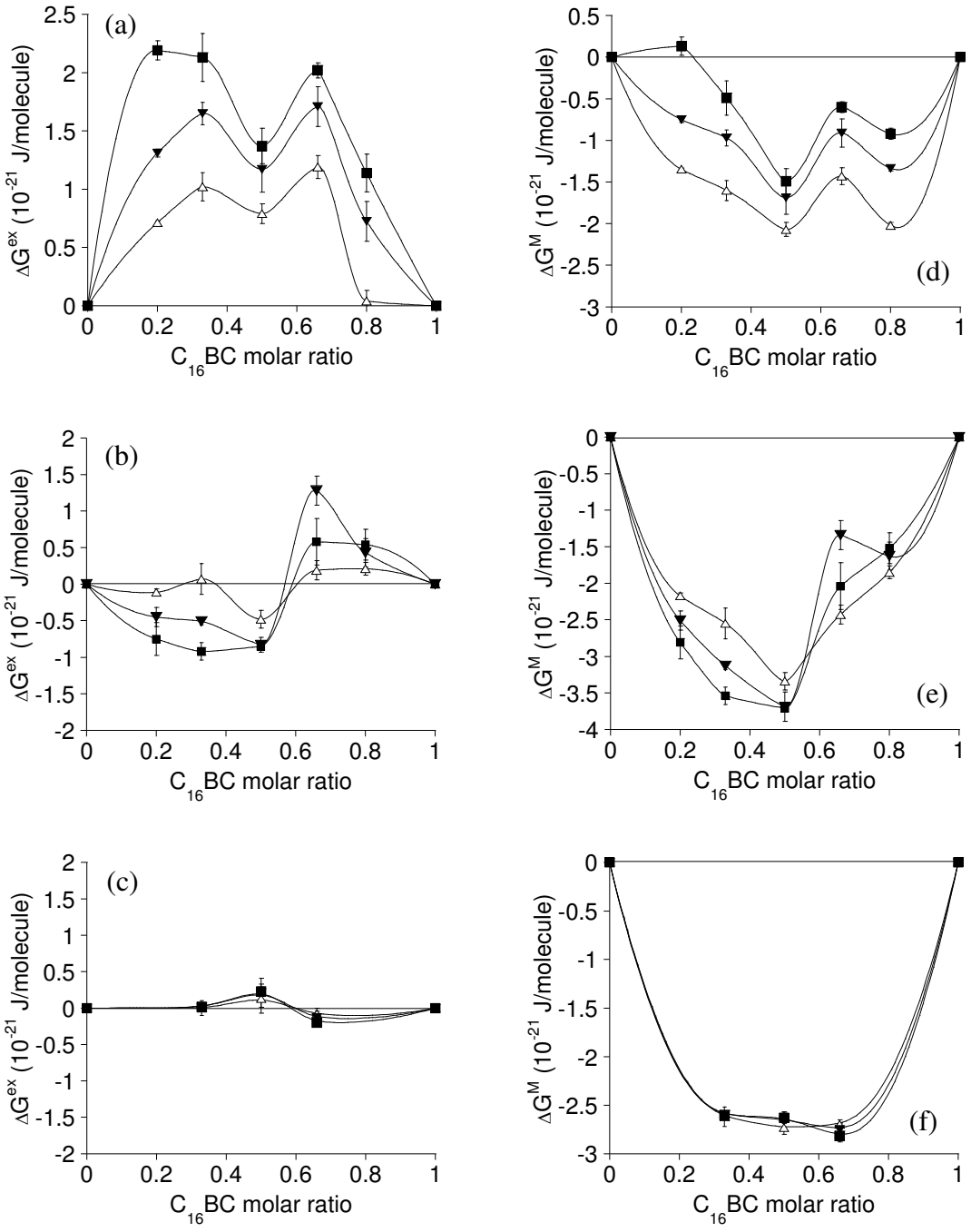

Figure 8: Excess free energy of mixing (ΔG^{ex}) and total free energy of mixing (ΔG^{M}) versus C_{16}BC molar ratio for the mixtures of C_{16}BC with POPC (a, d), SM (b, e) and CHOL (c, f)

at different Π ((Δ) 10 mN/m, (▼) 20 mN/m) and (■) 30 mN/m and at 25°C. Error bars are smaller than symbols in some cases.

The values of ΔG^M in Fig. 8d-f are negative for most of the C_{16}BC/POPC, all the C_{16}BC/SM and all the C_{16}BC/CHOL mixed monolayers implying that the mixing behaviour of C_{16}BC and each lipid is thermodynamically favorable within the whole range of composition and Π investigated. At a given Π, ΔG^M is more negative for C_{16}BC/SM and C_{16}BC/CHOL systems than for C_{16}BC/POPC. The mixed monolayers formed with SM and CHOL are more stable than those with POPC. For the C_{16}BC/POPC mixed monolayers, ΔG^M increases as Π increases, suggesting that the mixed monolayers become less stable at higher Π. At Π = 30 mN/m, C_{16}BC/POPC (1:1) mixed monolayer is the most stable monolayer (Fig. 8d). For the mixed C_{16}BC/SM monolayers, at a C_{16}BC molar ratio $\leq$ 0.5, the stability of the mixed C_{16}BC/SM monolayers increases with Π while at C_{16}BC molar ratio = 0.66, the stability decreases with increasing Π (Fig. 8e). The stability of C_{16}BC/CHOL is not influenced by Π (Fig. 8f).

3.4. Molecular modeling

The Hypermatrix method used for the molecular modeling is a "static" approach in the sense that the structure of the molecules is rigid[19]. Issues that might be sensitive to the way in which the chains are folded within the bilayer cannot thus be explored but this approach is particularly useful for investigating the nature of the interaction of drugs and other molecules with specific parts of the membrane and for estimating how the molecular area is affected by the interactions in the vicinity of the hydrophobic/hydrophilic interface.

The calculation of the interaction energy between one molecule of SM or POPC and one molecule of $C_{16}BC$ (Fig. 9) shows that these associations lower the total energy, as compared to pure lipids.

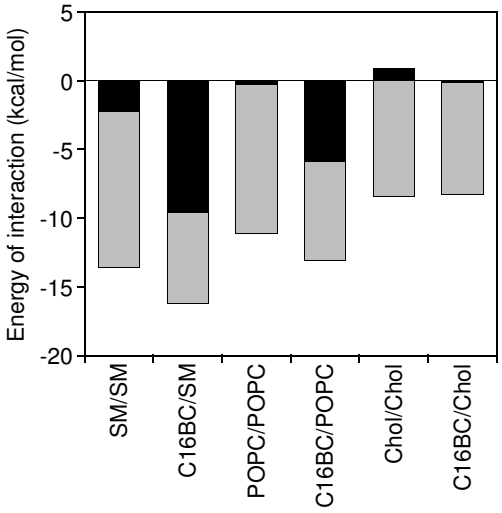

Figure 9: Total energy of interaction between the pure lipid molecules and between each lipid and $C_{16}BC$. Black and grey bars correspond to the polar and the hydrophobic contributions, respectively.

The most favorable interaction is obtained with SM which is in accordance with the ΔG^M values at $C_{16}BC$ molar ratio = 0.5. For SM and POPC, the polar contribution increases markedly when they interact with $C_{16}BC$ while the energy is mainly of hydrophobic origin for pure lipid self-association. In both cases, the PO_4^- of the lipid is close to the quaternary ammonium of $C_{16}BC$ (Fig. 10a and b) and can then interact electrostatically. Moreover, the ester moiety of $C_{16}BC$ is located at the level of the amide and the hydroxyl groups of SM (Fig. 10b). Formation of H bonds between these groups can also contribute to the polar component of the interaction energy.

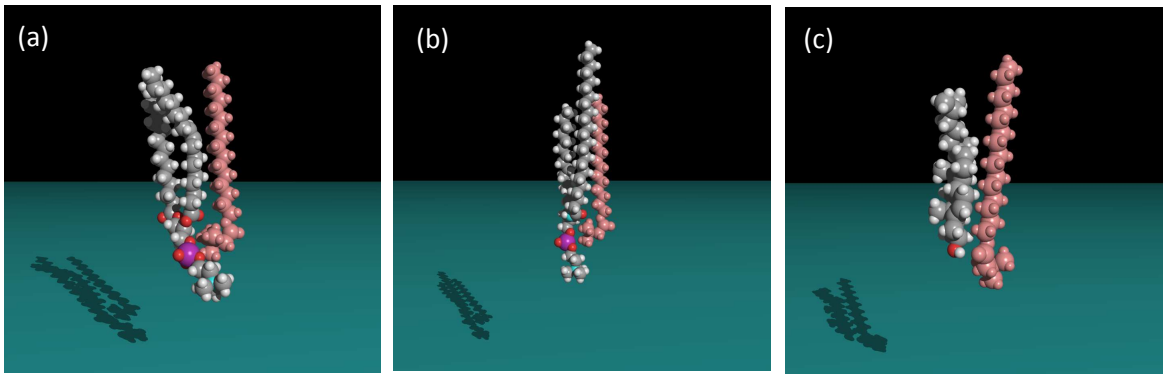

Figure 10: Molecular assembly of $C_{16}BC$ (in pink) with (a) POPC, (b) SM and (c) CHOL at an hydrophobic/hydrophilic interface.

In the case of CHOL, the interaction energies with $C_{16}BC$ or with itself are similar. Furthermore, the total calculated interfacial area occupied by both molecules in the assembly (94 Å²) is equal to the addition of the individual molecular areas (54 Å² for CHOL and 39 Å² for $C_{16}BC$) in accordance with our experimental results (Fig. 6c). The assembly of $C_{16}BC$ and CHOL does not appear to disturb their self arrangement at the interface. Their fitting shown in Fig. 10c can be considered as ideal. The OH group of CHOL is at the level of the $C_{16}BC$ ester bond, slightly decreasing the polar energy as compared to pure CHOL assembly and the sterol rings are parallel to $C_{16}BC$ alkyl chain, generating a large hydrophobic contribution to the interaction energy, as for pure CHOL.

4. General discussion and conclusion

The negative binding free energies ($\Delta G_D^{w\to b}$ – Table 1) of C_{16}BC for the lipid systems investigated indicate that the transfer of C_{16}BC from the aqueous phase into lipid bilayers is thermodynamically favorable. C_{16}BC surfactant (at a concentration near its CMC) is able to bind to lipid vesicles without compromising their integrity (Fig. 3). The increase of the surface pressure after the injection of C_{16}BC beneath the lipid monolayers also gives evidence of C_{16}BC penetration into all the lipid films investigated. Moreover, the exclusion pressure of C_{16}BC for the three lipids is above the presumed surface pressure of biological membranes, suggesting that C_{16}BC could be able to insert into *in vivo* mammalian plasma membranes containing high concentration of these three lipids.

Bilayer binding constant (K –Table 1) and monolayer equilibrium $\Delta\Pi$ (Fig. 4) values clearly show that the insertion of C_{16}BC is more important for the CHOL-containing films than for SM or POPC films. However, one could expect penetration to be favored into POPC as this lipid forms liquid-disordered lamellar phase[30] and monolayers less packed than those of CHOL or SM at a given surface pressure.[47,48] Our measurements indicate otherwise. It means that membrane fluidity is not the key feature to explain distinct C_{16}BC insertion between SM/CHOL and, SM and POPC.

Higher affinity of hexadecylphosphocholine, another quaternary ammonium surfactant with a 16 carbon atoms chain, for CHOL rather than POPC has also been previously reported.[49] This may be due to the fact that the CHOL sterol rings can fit nicely into the C_{16}BC alkyl-chain region and that its hydroxyl group helps to anchor the CHOL molecule at the C_{16}BC head–alkyl-chain interface as shown by the molecular modeling of their assembly (Fig. 10c). This suggests an ideal miscibility, i.e. a uniform distribution, of C_{16}BC and CHOL, and

corroborates the ideal behavior observed for the mixed C_{16}BC/CHOL monolayers (Fig. 7). However, the kinetic of their optimal mutual organization is slow as shown by the long stabilizing time of $\Delta\Pi$ observed in monolayer systems (Fig. 4). The very small value of ΔG^{ex} for C_{16}BC-CHOL monolayers seems to be inconsistent with the large $\Delta S_D^{w\to b}$ observed for the C_{16}BC-CHOL-SM bilayer. However, both parameters do not reflect the same phenomenon. In the bilayer experiments there is an adsorption and insertion stage while in the monolayer experiments, both molecules are spread simultaneously at the interface and no adsorption phenomenon occurs. The $\Delta S_D^{w\to b}$ value calculated from bilayer results takes into consideration the global interaction phenomenon comprising the adsorption, the insertion and the rearrangement of both components due to their strictly speaking interaction while the ΔG^{ex} calculated from monolayer data is restricted to the last step.

POPC has a larger head group than CHOL and cannot pack tightly due to head-tail mismatch, causing its tail group to be tilted even at high surface pressures. Moreover, the presence of a cis double bond in the hydrocarbon chain of POPC prevents their close packing and increases the intermolecular distance in the monolayer[50] as reflected in the area per molecule in the one-component monolayer (Fig. 6). The steric hindrance of the POPC molecule is high and insertion of C_{16}BC molecules between POPC molecules is less favorable. Similar results have been obtained for the interaction between stearic acid and DOPC.[51]

SM possesses a trans double bond located at the C_4–C_5 of its sphingosine backbone (Fig. 1b), and shows a tighter packing than POPC in its monolayer (A_c values from Fig. 6).[52,53] Hydrogen bonds between the amide or the hydroxyl of the acyl chain of SM and the carbonyl group of C_{16}BC can be formed, favoring the polar interaction between the molecules. Moreover, they can promote the van der Waals interactions between the alkyl

chains of C_{16}BC and of SM.[54] These interactions stabilize the monolayer as shown by their negative ΔG^M (Fig 8e) and the much higher Π_c for the mixed systems compared to the pure ones (Fig. 6b). However, with a C_{16}BC molar ratio > 0.5, ΔG^{ex} (Fig 8b) becomes positive and the interactions are thus more repulsive. Hydrogen bonds between the hydrophilic groups can explain the more favorable C_{16}BC interaction with the SM monolayer rather than with the POPC as highlighted by the computer modeling calculation and as reflected by the Π_e determined. In bilayers, this difference is not significant. A higher flip-flop rate of C_{16}BC from the outer to the inner leaflet of the POPC bilayer could compensate this effect.

In general conclusion, this study has shown that the interaction between C_{16}BC and lipids is not governed by the membrane fluidity and that the forces involved can have an origin other than purely electrostatic, confirming the hypothesis developed in our previous paper.[14] The primary insertion of C_{16}BC into lipid vesicles is mainly governed by an hydrophobic effect. Subsequently, a convenient geometrical arrangement between the lipid and C_{16}BC involving polar interaction is favorable for their association. A best arrangement between surfactin, a lipopeptide, and dipalmitoyl-phosphatidyl-ethanolamine (DPPE) molecules has also been shown to be at the origin of the stabilizing effect of surfactin towards DPPE monolayer.[55] The optimal matching between the surfactant and a lipid for which the packing parameter is not in favor of the formation of a bilayer as it is the case for CHOL, can also stabilize the lamellar structure. Hydrogen bonds between the surfactant and the lipid also contribute to the monolayer stabilization.

Although this study mainly provides basic insights about the interaction of C_{16}BC with each of the major membrane components taken individually, it may serve as a useful basis for understanding the interaction of C_{16}BC with real membranes or at least, with membrane

domains that might be highly enriched in a single component. It may be suggested that under physiological conditions, C_{16}BC can interact with the outer leaflet of mammalian plasma membranes without compromising their stability. The presence of rafts composed by SM and CHOL could be favorable to the interaction. However, it must be kept in mind that the interaction of a surfactant with a real membrane might be sensitive to many properties that reflect the mixing of membrane components such as average chain order, average area per lipid, local surface potential, and the lateral distribution of lipid components. Further investigations using ternary mixtures of the three lipids investigated as more representative models of mammalian plasma membrane have to be performed to assess our hypothesis. Moreover, molecular dynamic calculations taking into consideration the reorientation of the molecules about the bilayer normal and the fast acyl chain conformation changes could also provide additional information about the interaction of C_{16}BC in a dynamic environment, more representative of a real membrane.

Acknowledgments

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Supporting information

Enlargement of the Π-A isotherm of SM monolayer showing the change of slope at ~25 mN/m.

This information is available free of charge via the Internet at http://pubs.acs.org/.

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Caption legends

Figure 1: Chemical structure of (a) the hexadecylbetainate chloride ($C_{16}BC$) and of the three model lipids selected: (b) Sphingomyelin (SM), (c) palmitoyl-oleoyl-phosphatidylcholine (POPC), and (d) cholesterol (CHOL).

Figure 2: (a) Typical raw data of an ITC experiment. The peaks are related to the successive injections of 5µl of SM/CHOL LUV suspension at 1000 µM into a 30 µM solution of $C_{16}BC$ at 25°C. (b) The cumulative heats of binding ($\sum\delta hi$) as a function of the lipid concentration in the cell (C^0_L). (■) SM/CHOL, (Δ) SM, (▼) POPC. The solid line represents the best fit using the Eq 1. The buffer used was PBS at pH 7.4. Error bars are based on reproducibility over at least two independent measurements.

Figure 3: Particle size distribution prior to (full line) and after (dashed line) ITC experiments (a) POPC, (b) SM, (c) SM/CHOL. Inset: value of the mean hydrodynamic diameter (d_h) (average of three independent measurements).

Figure 4: Typical adsorption kinetic of $C_{16}BC$ at a clean air-water interface (o) or into lipid monolayers spread at 20 mN/m: (■) CHOL, (Δ) SM, (▼) POPC. The arrow indicates the moment at which injection occurred. $C_{16}BC$ is injected at a final concentration of 1.18 µM into the subphase (PBS pH 7.4; 25°C).

Figure 5: Surface pressure increase ($\Delta\Pi$) caused by the penetration of hexadecylbetainate chloride ($C_{16}BC$) into pure monolayers of (■) CHOL, (Δ) SM, (▼) POPC as a function of

their initial surface pressure (Π_i). C_{16}BC is injected beneath the lipid monolayer at a final concentration of 1.18 μM in the PBS subphase at pH 7.4 and 25°C. Error bars are smaller than symbols in some cases. The solid line represents the linear fitting of the data (R^2 = 0.98 and 0.99 for CHOL and SM respectively. For POPC, the linear fitting has been done for $\Pi_i \geq 10$ mN/m and R^2 = 0.98).

Figure 6: Surface pressure (Π)–molecular area (*A*) isotherms for C_{16}BC and lipid mixtures spread onto PBS subphase pH 7.4 at 25°C: C_{16}BC/POPC (a), C_{16}BC/SM (b) and C_{16}BC/CHOL (c).

Figure 7: Mean molecular area (A) versus composition plot for the mixtures of C_{16}BC with POPC (a), SM (b) and CHOL (c) at different Π ((Δ) 10 mN/m, (▼) 20 mN/m) and (■) 30 mN/m) at 25°C. Straight lines correspond to the ideal mixing behavior (Eq. 3). In some cases, the error bars are smaller than the symbols.

Figure 8: Excess free energy of mixing (G^{ex}) and total free energy of mixing (G^{M}) versus C_{16}BC molar ratio for the mixtures of C_{16}BC with POPC (a, d), SM (b, e) and CHOL (c, f) at different Π ((Δ) 10 mN/m, (▼) 20 mN/m) and (■) 30 mN/m and at 25°C. Error bars are smaller than symbols in some cases.

Figure 9: Total energy of interaction between the pure lipid molecules and between each lipid and C_{16}BC. Black and grey bars correspond to the polar and the hydrophobic contributions, respectively.

Figure 10: Molecular assembly of $C_{16}BC$ (in pink) with (a) POPC, (b) SM and (c) CHOL at an hydrophobic/hydrophilic interface.

Legend of Table

Table 1: Thermodynamic parameters for the binding of $C_{16}BC$ to LUV with different compositions at 25°C

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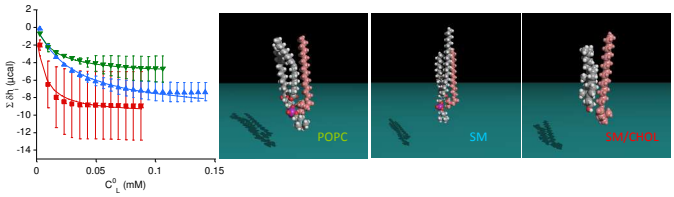

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