Spectrofluorimetric and Zeta potential studies of alkylbetainate chloride surfactants interaction with model membranes

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Abstract

The effects of cationic alkylbetainate chlorides, glycine betaine-based ester surfactants, on structural integrity of small unilamellar vesicles made of pure and mixture of palmitoyloleoyl phosphatidylcholine, palmitoyloleoyl phosphatidylglycerol, sphingomyelin and cholesterol are studied. The interaction is monitored by lipid vesicle permeabilization assessed by release of 8-hydroxypyrene-1,3,6-trisulfonic acid quenched by p-xylene-bis-pyridinium bromide and by zeta potential measurements after incubation of lipid vesicles in the presence of increasing concentrations of alkylbetainate chlorides at ambient temperature (25°C).

Results show that decylbetainate chloride and hexadecylbetainate chloride are less efficient in destabilizing lipid vesicles whereas dodecylbetainate chloride and in some extend tetradecylbetainate chloride are able to perturb model membrane integrity when reaching a critical concentration. The effect is more and less pronounced in zwitterionic palmitoyloleoyl phosphatidylcholine and cholesterol-containing (palmitoyloleoyl phosphatidylcholine/sphingomyelin/cholesterol) membranes than in negatively charged palmitoyloleoyl phosphatidylglycerol and mixture palmitoyloleoyl phosphatidylcholine / palmitoyloleoyl phosphatidylglycerol membranes. The kinetics of release are similar for each alkylbetainate chloride in both palmitoyloleoyl phosphatidylcholine and palmitoyloleoyl phosphatidylglycerol membranes. Measurements of zeta potential indicate that adsorption of alkylbetainate chloride to membranes is concentration and alkyl chain length-dependent, and attractive electrostatic interactions do not play a significant role.

Keys words: Lipid vesicles ; adsorption ; leakage ; surfactants ; alkylbetainate chlorides.

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1. Introduction

Biological membranes are of fundamental importance to living cells by serving as selective barriers for transport and boundaries for energy and information (1). The disruption of biological membranes by amphiphilic molecules has been a frequent focus for study because of its applicability to basic science as well as widespread human use (2). With the aim of studying interactions between surfactants and cell membranes, several approaches are possible. Living cells can be used or cellular membranes can be isolated and observe what happens in *situ* (3). However, natural membranes are entities with a great variety of lipids and proteins,

thus, in this case, only global phenomena can be investigated. To investigate a given phenomenon occurring at membrane level, the best choice is the use of membrane models (3). Of the different model systems, lipid bilayers (liposomes) as model membranes are generally studied to obtain information about the effect of toxic molecules like antibiotics, surfactants on the real membrane structure (4,5,6). To obtain reliable structural and thermotropic information on real membranes and to elucidate the molecular mechanisms of membrane specificity and permeabilization, mixtures of different lipids are applied in model investigations (7,8,9).

Several classes of molecules such as simple surfactants or amphipathic peptides perturb membrane permeability and induce the release of trapped material (10,11). Their presence can lead to the release of haemoglobin from erythrocytes, causing the death of the cell, or the release of a drug from a liposomal preparation. Interactions with surfactants represent one of the most widely investigated topics in the field of liposome research. In pharmaceutical technology, surfactants are increasingly important because of their ability to solubilize water-insoluble drugs (12). The general danger in using surfactants is their tendency to disrupt cell membranes. It is well known that most surfactants seem to bind to membranes even at low concentrations, which affects the membrane properties in many ways (13). Higher concentrations of surfactants lead to more drastic effects such as membrane lysis and fusion (14). Because of this potential use of surfactants in membrane chemistry, it is very important to study their effect on the membranes, especially on lipid bilayers which are drug delivery agents, at various concentrations.

There is a growing interest in the study of the physicochemical and biological properties of surfactants from renewable resources (plant and animal oils, micro-organisms) because of their characteristics, in particular their biodegradability and environmental compatibility which make their use more convenient as compared to surfactants from petrochemical source (15,16).

Alkylbetainate chlorides (C_nBC) are glycine betaine-based ester surfactants with foaming and dispersing properties (17,18). They have been found to be mild to the skin and are good candidates for cosmetic, pharmaceutical formulations (19,20). C_nBC (n = 10-16) have shown to penetrate strongly into pre-formed lipid monolayers of negatively charged lipids and of small head groups than into monolayer of zwitterionic phospholipid with bulky head groups (21).

The aim of the present work is to investigate the effects of C_nBC on model membranes of various composition and to get insight into the molecular details of their interactions with membranes. Two aspects of their membrane interaction are characterized. Their effects on membrane permeability are followed by the C_nBC-induced leakage of a fluorescent probe (HPTS) entrapped in lipid vesicles of different composition, using the fluorescence assay. Their binding to model membranes are characterized using the zeta potential measurements.

2. Materials and methods

Materials

Alkylbetainate chlorides (C_nBC) were synthesized in our laboratory. Their purity was checked by HPLC and NMR (22). The chemical structures of the C_nBC are shown in Fig. 1. Palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylglycerol (POPG), sphingomyelin (SM) and cholesterol (CHOL) were purchased from Avanti Polar Lipids (Alabaster, AL) and were used as received. Trisodium 8-hydroxypyrenetrisulfonate (HPTS) and p-Xylene-bis-pyridinium bromide (DPX) were purchased from Molecular Probes (OR, USA).

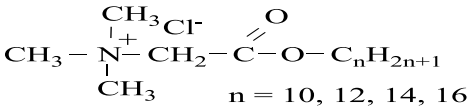

Fig. 1. Chemical structure of the alkylbetainate chlorides (C_nBC)

Methods

Preparation of lipid vesicles containing the complex HPTS-DPX

Small unilamellar vesicles (SUVs) were used in our experiments. The vesicles were prepared using the lipid hydration technique. First, POPC, POPG, POPC/POPG (70:30 mol %) or POPC/SM/CHOL (50:20:30 mol %) was dissolved in chloroform/methanol (2:1), and put in a 10 mL round-bottom flask. Chloroform/methanol was removed under vacuum by using a rotary evaporator resulting in a thin lipid film. The organic solvent traces were evaporated overnight. The dried thin lipid film obtained was hydrated for 1 h at 37°C with 1 mL of buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4), 12.5 mM HPTS in 45 mM NaCl and 45 mM DPX in 20 mM NaCl. Spontaneously formed multilamellar vesicles (MLVs) were subjected to 10 min periods of sonication to form

SUVs which were then centrifuged for 10 min at 2000g. SUV suspensions were passed through a Sephadex G-75 column to remove unencapsulated material. The concentration of the SUV suspensions was determined by the phosphorus analysis (23).

Leakage experiments

Vesicle leakage was measured by the assay of Ellens et al. [24] based on the quenching of HPTS by DPX encapsulated in the aqueous phase of the SUVs prepared before (see Section 2.2.1). The quenching of HPTS by DPX decreases when leakage of vesicle content occurs which increases the fluorescence of HPTS. Perkin-Elmer LS-50B fluorimeter was used at the excitation and the emission wavelengths of 450 and 512 nm, respectively. The release of fluorescent HPTS was expressed as a percentage of the maximum fluorescence intensity measured after adding 0.25% (v/v) Triton X-100 into the SUV suspensions. Control experiments were carried out by adding the buffer to the SUV suspensions at the same surfactant volume. The percentage of release is defined as:

$$\text{HPTS release (\%)} = \frac{F - F_0}{F_t - F_0} x100 \qquad \text{Equation 1.}$$

where F is the fluorescence intensity induced by the addition of the surfactant; F_0, is the fluorescence intensity of the intact vesicles and F_t is the fluorescence intensity corresponding to the complete release induced by the addition of Triton X-100. Each experiment was conducted with a separate control experiment for that vesicle preparation. All experiments were repeated in triplicate and averaged.

Zeta potential measurements

The zeta potentials of the lipid vesicles prepared in Tris buffer solutions were measured by Doppler electrophoretic light scattering analysis using a Backman Coulter Delsanano C (A 53878) instrument equipped with a 30 mW He-Ne laser (λ = 632.8 nm) and dedicated standard cell. The laser light, illuminating the moving colloid particles, provides their electrophoretic mobility in an applied electric field. The conversion of results from electrophoretic mobility to zeta potential was automatically carried out by the instrument using Smoluchowsky's equation:

$$\zeta = 4\pi\eta\mu / \varepsilon$$ Equation 2.

where μ is the electrophoretic mobility, η the viscosity of the solvent, and ε its dielectric constant. The lipid concentration used was 12.5 μM, as in the leakage experiments at 25 °C. Each data point represents the mean of three independent measurements with SD.

3. Results

In order to follow the intracellular fate of lipid vesicles, an endocytosis assay based on the dye HPTS has been applied. HPTS is a well-established, membrane-impermeant and pH-dependent fluorophore, which emits maximally at 512 nm [25]. HPTS was loaded into lipid vesicles as the water-soluble (but fluorescence quenched) complex, HPTS-DPX. When HPTS-DPX leaks from lipid vesicles, it dissociates into free HPTS and DPX, increasing the HPTS fluorescence.

Leakage of lipid vesicles

In order to investigate whether C_nBC impart a detrimental effect on liposomal membrane integrity, SUVs made of POPC, POPG, POPC/POPG (70:30 mol %) and POPC/SM/CHOL (50:20:30 mol %) and encapsulating the HPTS-DPX complex in their internal aqueous space are assayed spectrofluorometrically in the presence of increased concentrations of C_nBC. In the absence of C_nBC, no leakage of HPTS-DPX from the SUVs is observed. The fluorescence intensity in the absence of C_nBC is low and is considered as a control experiment. Fig. 2 shows the % leakage induced by the C_nBC in SUVs after 15 min of incubation at 25°C, as a function of C_nBC-to-lipid molar ratio. C_nBC can be devised into two groups. The first group composed of C_{10}BC and C_{16}BC which induce less than 20 % of HPTS leakage into the buffer outside the SUVs in all the model membranes at all the C_nBC-to-lipid molar ratio (from 0.1 to 20) investigated. Whereas, the second group composed of C_{12}BC and C_{14}BC induce a leakage of more or less 20 % at C_nBC/lipid ≤ 2.5 but more than 20 % at C_nBC/lipid > 2.5. In contrast, the leakage induced by C_{12}BC and C_{14}BC at high C_nBC/lipid (> 2.5) is important in zwitterionic POPC vesicles and mixture POPC/SM/CHOL vesicles than in negatively charged POPG and mixture POPC/POPG model membranes (Fig. 2). Note that C_{12}BC induces a higher degree of leakage in all the membranes (with a maximum at C_{12}BC/lipid between 7 and 10) than C_{14}BC. The addition of larger amounts of C_{12}BC (C_{12}BC/lipid >7 or 10) and in same cases of C_{14}BC, depending on membrane composition, leads to a decrease of the % leakage. Although CHOL

decreases membrane-destabilizing effect [26], in this study, the cholesterol-containing membrane seems to be more perturbed by C_{12}BC than the other model membranes investigated.

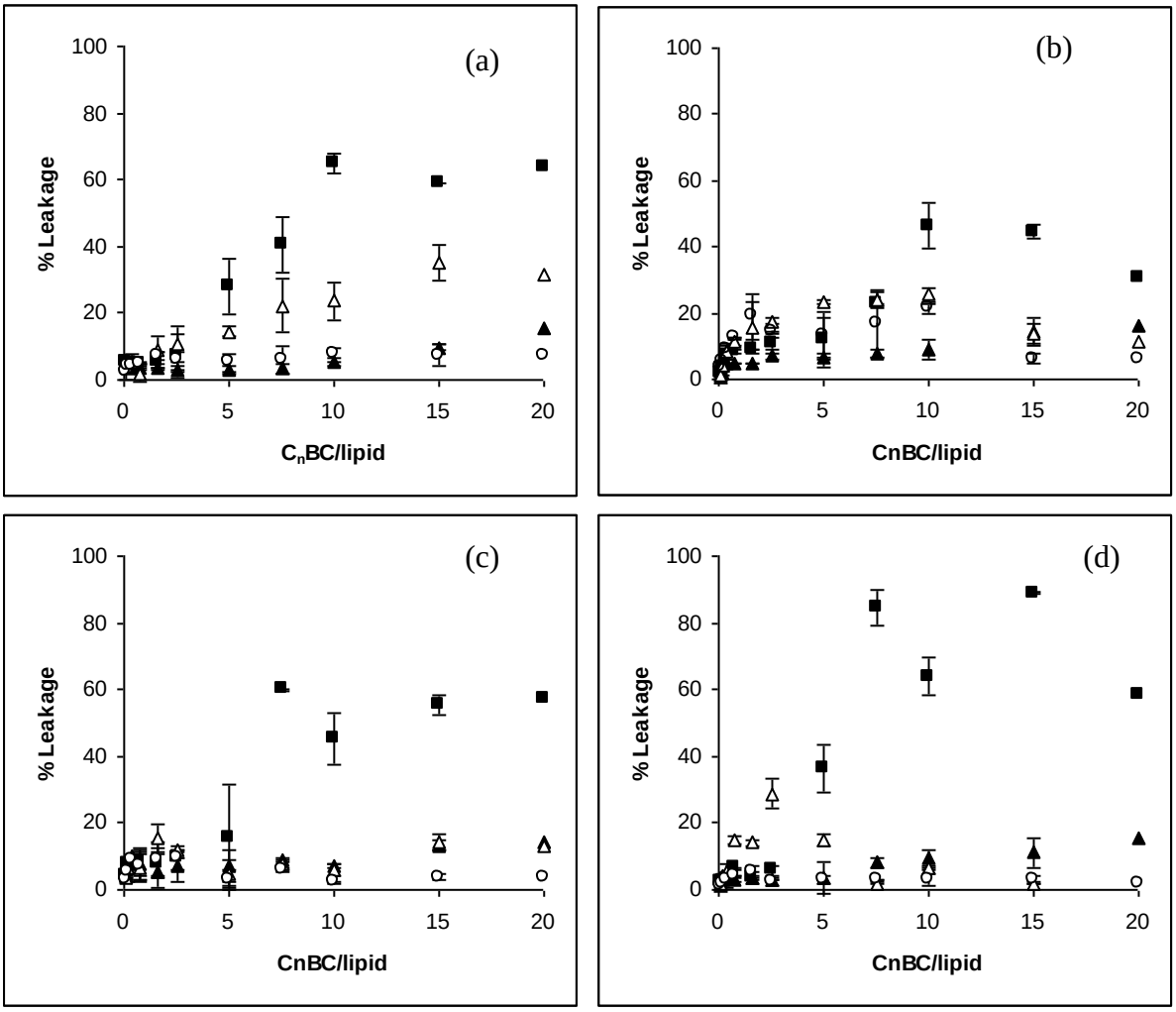

Fig. 2. HPTS leakage from SUVs induced by C_nBC [C_{10}BC (▲),C_{12}BC (■),C_{14}BC (Δ) and C_{16}BC (○)]. The lipid composition is (a) POPC, (b) POPG, (c) POPC/POPG (70:30) and (d) POPC/SM/CHOL (50:20:30). The HPTS leakage percent is measured 15 min after adding different amount of the C_nBC, and plotted as a function of C_nBC-to-lipid molar ratio. The HPTS fluorescence is recorded at an ambient temperature (25°C). Results are from a representative experiment, and are means of a least triplicate analyses ± S.D. (error bars are smaller than markers in some cases).

Kinetics of leakage

The release kinetics from zwitterionic POPC and negatively charged POPG vesicles with C_nBC at C_nBC-to-lipid molar ratio of 10 (where the maximum of HPTS release is observed) are monitored over 15 min at 25°C. Fig. 3 shows the % of HPTS released as a function of time in buffer. The data suggest a hyperbolic dependency of leakage versus time for C_{12}BC and a almost linear profile for C_{10}BC, C_{14}BC and C_{16}BC for POPC and POPG vesicles. C_{12}BC causes a higher degree of leakage in POPC vesicles (63 %) than in POPG vesicles (47%) while the degree of leakage is similar and of the same order of magnitude lower for C_{14}BC (~24 %) and very lower for

C_{10}BC and C_{16}BC (~7 and 10 %, respectively) in both model membranes. These results are unexpected and suggest that the interactions between C_nBC and these two model membranes in the experimental conditions follow the same mechanisms.

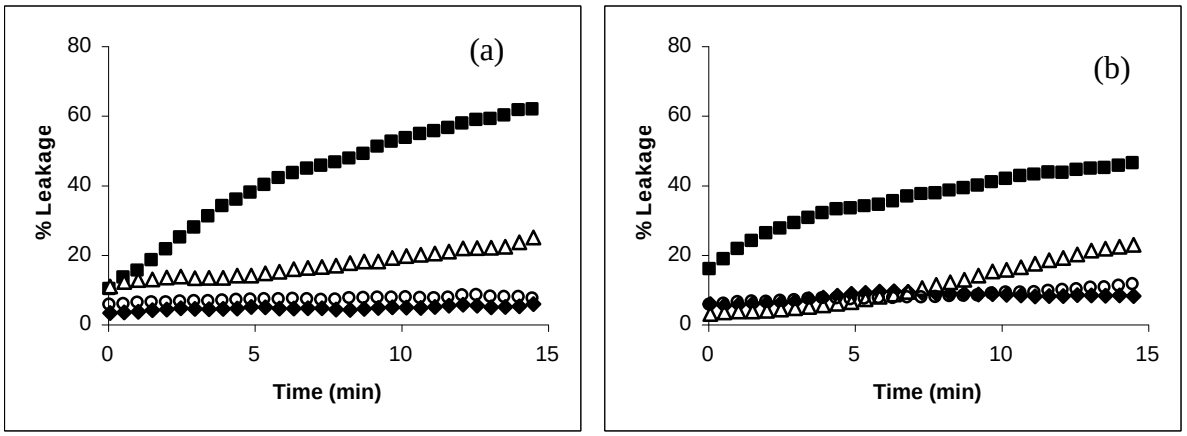

Fig. 3. Time-dependence of HPTS leakage from (a) POPC and (b) POPG SUVs due to addition of C_nBC [C_{10}BC (♦), C_{12}BC (■),C_{14}BC (Δ) and C_{16}BC (○)] at surfactant to lipid molar ratio of 10. The HPTS released was plotted as a function of time. The buffer is 10 mM Tris, 150 mM NaCl (pH 7.4). The HPTS fluorescence was recorded at an ambient temperature (25°C).

Surface charges of lipid vesicles (Zeta potential)

The zeta potential describes the electric potential of particles in aqueous solution at the shear plane close to their surface and is an indicator for accessible surface charges (27, 28). To account for the electrostatic effects of the interaction between C_nBC and lipid vesicles, zeta potential values of zwitterionic POPC and negatively charged POPG SUVs are measured at some surfactant-to-lipid molar ratios as used in the leakage experiments for C_{10}BC, C_{12}BC and C_{16}BC. Fig. 4 shows zeta potential values for POPC and POPG vesicles when adding increasing concentrations of different C_nBC. The results show that zeta potential values are negative for POPC (-16.30 ± 0.06 mV) and POPG (-32.65 ± 3.60 mV) SUVs. Zeta potential values are also negative for POPC/POPG and POPC/SM/CHOL model membranes (data no shown), as observed by others [29]. The differences can be due to the buffer and the ionic strength of the solutions used. The addition of C_{10}BC causes a slight but insignificant increase of zeta potential (-6.78 mV) in POPC SUVs (Fig. 4a). Also, C_{10}BC gives rise to a zeta potential increase to -22 mV only in POPG SUVs (Fig. 4b). C_{10}BC is incapable to compensate the charge density of POPC and POPG SUVs even at C_{10}BC/lipid = 20. However, C_{12}BC and C_{16}BC influence the zeta potential of both model membranes and a charge reversal is observed at relatively larger surfactant-to-lipid molar ratios.

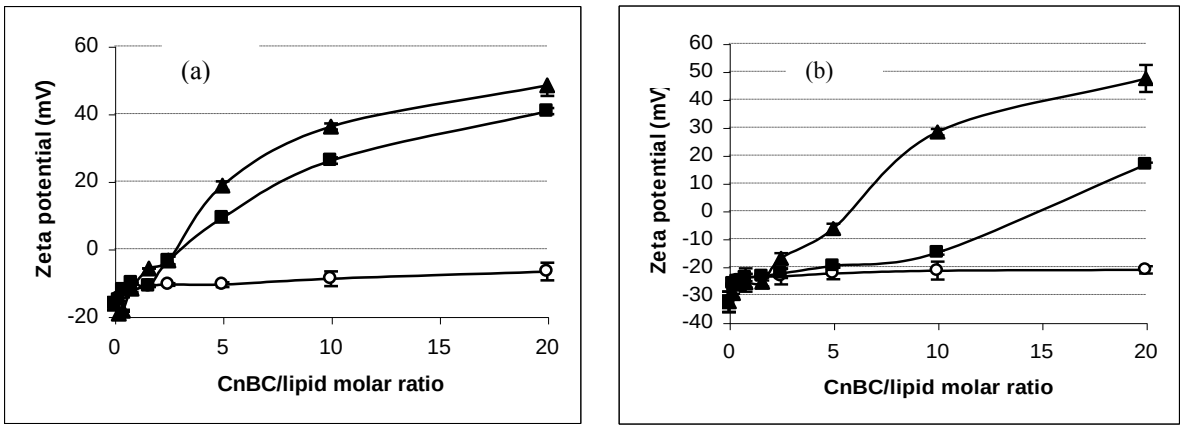

Fig. 4. Effects of different C_nBC on the zeta potential of POPC (a) and POPG (b) SUVs. Different concentrations of $C_{10}BC$ (○), $C_{12}BC$ (■) and $C_{16}BC$ (▲) are added to a 12.5 mM solution of SUVs in a 10 mM Tris buffer, 150 mM NaCl, pH 7.4. Measurements are performed at 25°C. Results are represented as mean and standard deviation of three independent experiments.

4. Discussion

The results presented in this work provide detailed molecular information about the mechanism of membrane permeabilization and adsorption by/of cationic alkylbetainate chlorides (C_nBC, n = 10-16). The relatively simple system of fluorescent HPTS leakage from lipid vesicles and the description of their surface charges allow the careful determination of the kinetics of the process of leakage and of the adsorption, as well as the effect of membrane lipid composition. Several steps are involved in the mechanism of leakage of lipid vesicles by surfactants. These steps include the adsorption of the surfactant to the outer leaflet of the membrane, flip-flop to the inner leaflet, and finally the proper intercalation between the lipid molecules (26).

No significant % leakage is observed with $C_{10}BC$ and $C_{16}BC$ at surfactant-to-lipid molar ration from 0.1 to 20 for all the model membranes investigated in this study (Fig. 2). The weak % leakage found with $C_{10}BC$ is in accordance to the zeta potential data showing a very weak binding to zwitterionic POPC and to the negatively charged POPG SUVs although the surface charges of the membranes are negative (Fig. 4). In contrast to $C_{10}BC$, $C_{16}BC$ is highly bound to both POPC and POPG membranes according to the data of the zeta potential in Fig. 4. This suggests that $C_{16}BC$ molecules do not perturb the membrane structure and seem to stabilize it. Thus, the weak % leakage observed with $C_{10}BC$ and $C_{16}BC$ in all cases must be the result of the increase in the

phospholipid hydrocarbon chain conformational disorder and the perturbation of the packing of the lipid molecules (26,30).

At low surfactant-to-lipid molar ratios (<1.6), C_{12}BC and C_{14}BC behave similarly to C_{10}BC and C_{16}BC. However, once a critical threshold concentration occurs, C_{12}BC and C_{14}BC induce leakage to the investigated model membranes. The % leakage reaches a maximum in the case of C_{12}BC (C_{12}BC/lipid = 7-10) and in the same in some extend in the case of C_{14}BC (C_{14}BC/lipid = 2.5-10), depending on membrane composition, and decreases at larger C_{12}BC/lipid or C_{14}BC/lipid for all the model membranes. It is interesting the finding that the surfactant-to-lipid molar ratio to initiate a substantial leakage with C_{12}BC and C_{14}BC in all the membranes apparently does not vary with the composition of the membrane, and is close to 7-10 and 5-7.5 for C_{12}BC and C_{14}BC, respectively. This membrane perturbation effect by C_{12}BC and C_{14}BC is in the following order: POPC/SM/CHOL > POPC > POPC/POPG > POPG (Fig. 2).

The leakage of dye from lipid vesicles can be induced either by making channels in them (pore-formation) or by lysis of the lipid vesicles (31). The latter is unlikely since the vesicles exhibit only partial leakage in presence of high concentrations of either C_{12}BC or C_{14}BC.

C_nBC bear the same bulky head group but differ by the length of alkyl chain (Fig.1). Generally, the release of fluorescent probe from lipid vesicles is proportional to the alkyl chain length of the membrane-disrupting agents, either surfactants, drugs or peptides (31,32). Indeed, the molecular basis of the relatively greater potency of C_{12}BC and in same extend C_{14}BC to perturb the membranes at a critical threshold concentration compared to C_{16}BC can be due to the relatively weak hydrophobic interactions between these surfactants and the lipids once incorporated into the lipid vesicle core. This phenomenon can result in transient pore-formation during an initial bilayer perturbation period, followed by a transient restabilization of the surfactant/lipid bilayer structure (33). The leakage mechanism of the HPTS fluorescent probe from the aqueous interior phase of the lipid vesicle into the buffer outside the SUVs, induced by the C_nBC cationic surfactants can be schematically represented as in Fig. 5 below. The addition of larger surfactant amounts leads to a decrease of the % leakage. This effect has already been reported for the perturbation of POPC, POPC/POPG and POPC/CHOL membranes by cetylpyridinium chloride (34). This can be due to the accumulation of surfactants

on the bilayer surface which obstruct partially the pores formed so that dye leakage is reduced, since a translocation of surfactant molecules across the membranes increases vesicle leakage (35).

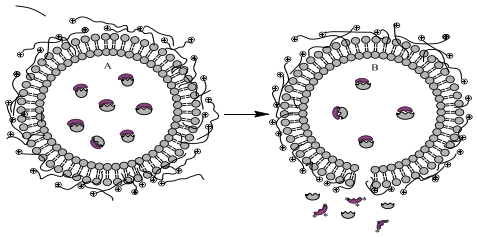

Fig. 5. Schematic representation of the leakage mechanism of lipid vesicles by C_nBC. (A. C_nBC bind to lipid vesicles and incorporate their hydrophobic alkyl chain into the lipid vesicle core. B. Pore-formation followed by leakage of complex HPTS-DPX and it dissociation to HPTS fluorescent probe and DPX quencher).

Comparatively to previous works on permeability of lipid vesicles by quaternary ammonium compounds, no release of calcein from POPC large unilamellar vesicles has been observed for dodecyltrimethylammonium chloride at surfactant/lipid molar ratio of 10 (32) whereas the incubation surfactant/lipid molar ratio to reach 50% of calcein release in POPC vesicles has been 1 and 36 for hexadecyltrimethylammonium chloride (32) and hexadecyltrimethylammonium bromide (36), respectively. This suggests that the structure of the surfactant, including alkyl chain and polar head group (size (37), counterion (38,39)), electrical charge distribution in surfactant molecules (40)…, influence the physico-chemical properties of surfactant systems.

It is known that CHOL increases the motional order of the phospholipid acyl chains, resulting in a tighter lipid packing (41,42) and thus decreases membrane-destabilizing effect (26). In this work, POPC/SM/CHOL and POPC/POPG membranes seem to be more perturbed by $C_{12}BC$ than POPC and POPG membranes. Simularly, the lack of influence of the membrane cholesterol content has also been reported by others (36,43,44,45). This can be explained by the fact that mixtures in general are more sensitive than simple lipids (46). Moreover, we have reported before that C_nBC penetrate more into CHOL liquid-condensed monolayers than into POPC liquid-expensed one at 30 mN/m (21), as expected from the complementary cone molecular shape of C_nBC and CHOL which are inverted cone-shape molecules and cone-shape molecule, respectively. In the case of POPG membrane, the translocation of cationic surfactants across the membrane can be limited by the electrostatic interactions between the positive charge of the surfactant's head groups and the negative charge of the phosphate groups in POPG, thereby stabilizing the vesicle permeability barrier (47).

For each C_nBC, the kinetics of leakage present the same profiles for both zwitterionic POPC and negatively charged POPG membranes (Fig. 3). These resemblances can be associated to the same mechanisms of interactions between C_nBC molecules and both model membranes. Although C_nBC head groups are positively charged and the surface charges of POPC and POPG membranes are negative, each C_nBC binds almost equally to both membranes and the binding seems to be dependent on the alkyl chain length of the C_nBC according to the results from the zeta potential measurements in Fig. 4. This behaviour is unexpected since the results of our previous work (21) have shown that C_nBC injected into the water subphase (pH 5.7) penetrate more into the negatively charged dipalmitoylphosphatidic acid and dipalmitoylphosphatidylserine monolayers and the penetration has not seemed to depend on the alkyl chain length of the C_nBC. This indicates that electrostatic interactions play an important role in their affinity to lipid monolayers in water. In contrast, the affinity of C_nBC to lipid bilayers in buffer (see section 2.1) is not affected by the negative surface charge of the lipid bilayers and seems to depend on concentration and alkyl chain length of the C_nBC, as revealed by the data from the zeta potential (Fig. 4) showing no membrane saturation even at larger surfactant's concentration. This suggests that the binding of C_nBC to membranes is likely governed by hydrophobic interactions and therefore the attractive electrostatic interactions do not play a significant role. This behaviour can result from the screening of C_nBC's head groups by buffer's anions as it is known, in fact, that the higher ionic strength decreases the electrostatic interactions via charge screening (46). Once incorporated into the bilayer, C_nBC molecules likely interact with the surface charge carried by the lipid head groups of the membrane, as suggested by Marcotte et al. (34) and by Denyer et al. (48).

Conclusion

This study provides new insights into the effects of the molecular details of alkylbetainate chloride surfactants (C_nBC, n = 10-16), glycine betaine-based ester surfactants on their interactions with lipid bilayers. C_{10}BC and C_{16}BC seem not to perturb membrane integrity because the former binds very weakly to membranes whereas the latter although binds highly to membranes, stabilize them by hydrophobic interactions. In contrast, C_{12}BC and in some extend C_{14}BC are able to perturb the membrane integrity at critical concentrations depending on membrane composition due to their relatively weak hydrophobic interactions with membrane's lipids. Each C_nBC has similar kinetics of leakage in both zwitterionic POPC and negatively charged POPG membranes. The

binding of C_nBC to membranes appears to increase with increasing surfactant's concentration and alkyl chain hydrophobicity, attractive electrostatic interactions playing a minor role.

Further research needs to be carried out on the interactions between C_nBC and biological membranes (e.g. human erythrocytes and bacteria) to get an overall view of their membrane activities since apparent contradictions have been pointed out between the absence of leakage observed when some quaternary ammonium surfactants are added to lipid model membranes and their bactericidal effects (49,50,51). C_nBC are potential candidates for human consumption's products, for instance in cosmetics, pharmaceuticals, or as food additives. Nevertheless, their application to this aim needs the proper characterization of their possible side toxic actions.

Abbreviations

C_nBC: Alkylbetainate chlorides; C_{10}BC : decylbetainate chloride; C_{n2}BC : dodecylbetainate chloride; C_{14}BC : tetradecylbetainate chloride; C_{16}BC : hexadecylbetainate chloride; POPC: palmitoyloleoyl phosphatidylcholine; POPG: palmitoyloleoyl phosphatidylglycerol; SM: sphingomyelin; CHOL: cholesterol; HPTS: Trisodium 8-hydroxypyrene-1,3,6-trisulfonic acid; DPX: p-Xylene-bis-pyridinium bromide; MLV: multilamellar vesicle; SUV: small unilamellar vesicle.

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