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Effect of ionic and neutral surfactants on the properties of phospholipid vesicles: investigation using fluorescent probes

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Abstract

The effect of triton X-100, sodium dodecyl sulphate and cetyl trimethyl ammonium bromide on the physical properties of dimyristoyl phosphatidyl choline liposome was studied in the sublytic concentration range of the detergent, using the different fluorescence probes 8-anilino-1-naphthalene sulphonate, 1,6-diphenylhexatriene and 1-naphthol. Depending on the nature and concentration of the detergent, and the position of the hydrophilic and hydrophobic parts of different detergents in liposomes, the fluidity and permeability of the bilayers were affected. The fluidity decreases at very low concentrations of detergent owing to an improvement in the packing density of phospholipid molecules. The extent of changes in these properties was found to be dependent on the phase state of liposomes, being greater in the solid gel phase than in the fluid liquid crystalline phase. Also, the thermotropic phase transitions of phospholipid bilayers are affected moderately by neutral surfactants and greatly by charged surfactants owing to an alteration in the water of hydration of the lipid molecules. © 1997 Elsevier Science S.A.

Keywords: ANS; CTAB; DMPC lipsomes; DPH; Fluorescence probes; 1-Naphthol; SDS; Surfactants; Triton X-100

1. Introduction

Detergents are widely used as molecular tools in membranology and membrane mediated drug delivery [1]. This includes disintegration of biomembranes to mixed micelles [2], reconstitution of membrane proteins and lipids [3] to functional supramolecular structures (vesicles), and preparation of homogeneous lipid vesicles of controlled sizes [4]. In pharmaceutical technology, surfactants are increasingly important because of their ability to solubilize water-insoluble drugs [5]. 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 [6]. Higher concentrations of surfactants lead to more drastic effects such as membrane lysis and fusion [7]. During drug delivery to the target tissue, liposomes interact with serum containing amphipathic surfactant-like substances. These molecules might intercalate into the bilayer, altering the packing of the bilayers and increasing the permeability of the vesicles to any entrapped compounds [8]. Because of this potential use of detergents in membrane chemistry, it is very important to study their effect on the membranes, especially on phospholipid bilyers which are drug delivery agents, at various concentrations.

The biotechnological application of addition of surfactants to liposomes is directed towards increasing the entrapped volume for efficient loading of the drug and liposome fusion. Helenius and Simons [9] proposed that on increasing the amount of surfactant added, the phospholipid bilayers are converted to mixed micelles in three steps: (i) an equilibrium distribution of the detergent between the lipid and the water phase exists in stage I, (ii) coexistence of mixed bilayer and mixed vesicles occurs in the second step and (iii) mixed bilayers are transformed progressively into mixed micelles as the detergent concentration increases until all of the bilayers have disappeared. On the addition of surfactants, differential scanning calorimetric studies show that the thermotropic phase transition of phospholipid bilayers is affected [10]. This effect might be due to vesicle solubilization as well as binding of surfactants to the bilayers. Detailed literature information exists about step (ii), because of its importance from a mechanical as well as experimental point of view [11–13]. Stage I is very important as it will give information about the physical properties of surfactant entrapped intact liposomes. Although scanty reports on stage I are available for some neutral surfactants [14], a systematic study of the effect of binding of ionic and neutral surfactants in stage I has not been done.

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In the hope of shedding some light on this, we have studied the changes in the properties of DMPC (which is a well characterized liposome) on the addition of neutral surfactant triton X-100, anionic surfactant sodium dodecyl sulphate (SDS) and cationic surfactant cetyl trimethyl ammonium bromide (CTAB), at a very low surfactant concentration (stage I) when the membrane is intact. The studies were done using the standard fluorescence probes 8-anilino-1-naphthalene sulphonate (ANS), a polarity probe [15], 1,6-diphenylhexatriene (DPH), a polarization probe [16], and 1-naphthol, an excited state acid probe [17].

2. Experimental

The probes DPH (Sigma), ANS (Sigma) and 1-naphthol were used after purification by recrystallization in appropriate solvents and checking their melting points. The surfactants CTAB and SDS were purified by recystallization from methanol. Dimyristoyl phosphatidyl choline (DMPC) used for liposome preparation was obtained from Sigma and its purity was checked by looking out for a single spot in TLC. The solvents used were doubly distilled.

2.1. Liposome preparation [18] and labelling [19]

DMPC was dissolved in chloroform–methanol 2:1 v/v at the desired molar ratio. The solution was evaporated to dryness under nitrogen, and left under vacuum for a sufficient time to remove any residual solvent, followed by addition of 50 mM aqueous sodium chloride to yield a final concentration of 0.1 mM phospholipid. Freshly prepared multilamellar vesicles (MLVs) were used for all the experiments. The surfactants interact in much the same way with the hydrophobic core of both sonicated and non-sonicated liposomes [20], and as our studies were on the binding of surfactants, MLVs only were used.

Labelling of fluorescent probes on liposomes was achieved by two methods: (i) adding a measured amount of dye to the lipid solution, before preparation of liposomes, in a molar ratio yielding a final lipid/probe ratio of 100 for DPH and ANS, and around 75 for 1-naphthol, such that the probe is directly incorporated into the membrane; (ii) adding an unlabelled liposome suspension to an aqueous dispersion of DPH in tetrahydrofuran [19], aqueous solutions of ANS and 1-naphthol. After addition of the probe, the solution was allowed to equilibriate for about an hour and was checked for constancy in the fluorescence intensity with time. Liposomes labelled by both the methods were found to give the same sort of results. For each preparation, a control solution containing the same lipid components but lacking the probe was prepared under identical conditions to serve as a blank.

2.2. Incorporation of surfactants into liposomes

Surfactants were incorporated into liposomes by (i) adding a definite volume from a stock surfactant solution in chloroform to the lipid solution to achieve molar concentrations of surfactants varying from 0 to 5 mol% of the lipid concentration before preparation of the liposome; (ii) adding a definite volume of surfactant stock in water to the liposomes which have been formed and allowing the solutions to equilibriate for 2 h before the experiment. The results obtained from both methods were comparable. By keeping the lipid concentration at 0.1 mM, the surfactant concentrations were varied from 5 to 50 μM, well below the cmc of all three surfactants [21a]. Because the partition coefficients for the uptake of surfactants by liposomes have been shown to be very high at low concentrations [1], it is expected that the added surfactants would be almost completely bound to the lipid bilayer.

2.3. Turbidity measurements

The turbidity of various mol% surfactant entrapped vesicles was measured by taking the absorbance at 420 nm [14]. No appreciable change in the absorbance was observed in the presence of less than 5 mol% of surfactant added to the liposome. This indeed shows that the liposome is intact and does not undergo any solubilization in the presence of the various mol% surfactants added. Since solubilization of the liposome occurs above 5 mol%, the concentration range of surfactants is confined to 0–5 mol% of the lipid concentration.

2.4. Polarization measurements

Fluorescence polarization measurements were carried out by the conventional method [22] of measurements of $I_{\parallel}$ and $I_{\perp}$, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities detected through a polarizer oriented parallel and perpendicular to the direction of polarization of the excitation beam. The fluorescence polarization (P) of the probe was obtained using the following relationship [22]:

$$P=\frac{(I_{\parallel}-I_{\parallel s})-(I_{\perp}-I_{\perp s})}{(I_{\parallel}-I_{\parallel s})+(I_{\perp}-I_{\perp s})}$$

where 's' stands for the scattering component.

From the polarization values of DPH, the microviscosity can be calculated using the modified Perrin equation [22] for DPH:

$$\bar{\eta}=\frac{2P}{0.46-P}$$

as the lifetime of DPH does not change appreciably owing to the binding of surfactants to liposomes [14]. Corrections for the scattering were obtained with dye-free controls. The error contribution from the control was found to be less than 0.5% of the polarization value.

2.5. Instrumentation

Absorption, turbidity and fluorescence measurements were taken with a Hitachi 2108 spectrophotometer and Hitachi

F4500 spectrofluorimeter respectively. Polarization measurements were taken with polacoat filter polarizers. The temperature was controlled with a thermostat bath. A deviation of less than 0.5°C in the recorded temperature was estimated for all the systems measured.

3. Results

3.1. Changes in the fluorescence properties of ANS

The binding of the fluorescent probe ANS is studied to gain information about the changes in the structure and properties of the polar headgroup region of liposome, on the addition of different surfactants. ANS is not fluorescent in water, and on the addition of liposome suspension to a known concentration of ANS in water a tremendous enhancement in the fluorescence was observed. The fluorescence intensity of ANS is measured at 478 nm (excitation at 360 nm). The surfactant induced changes in the fluorescence intensity of ANS bound to liposomes, on increasing the concentration of triton X-100, SDS and CTAB, are given in Fig. 1. The fact that the fluorescence of membrane bound ANS changes on addition of surfactants indicates the incorporation of surfactants to the liposomes. The addition of triton X-100 (Fig. 1(A)) increases the fluorescence of ANS up to 1.0 mol%, after which the emission intensity starts to decrease. The addition of increasing amounts of negatively charged SDS (Fig. 1(B)) decreases the fluorescence of ANS. When positively charged CTAB is added, the fluorescence of ANS increases continuously up to 5 mol% (Fig. 1(C)). The same trend is observed on the addition of different surfactants, in the gel phase, near the phase transition temperature of the liposome, and also in the liquid crystalline phase.

3.2. Changes in the fluorescence properties of 1-naphthol

1-naphthol is an excited state acid and, depending on the microenvironment, the excited state proton transfer (ESPT)

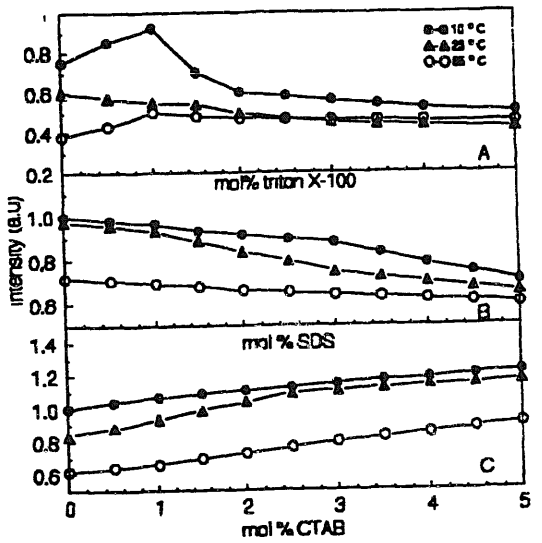


Fig. 1.

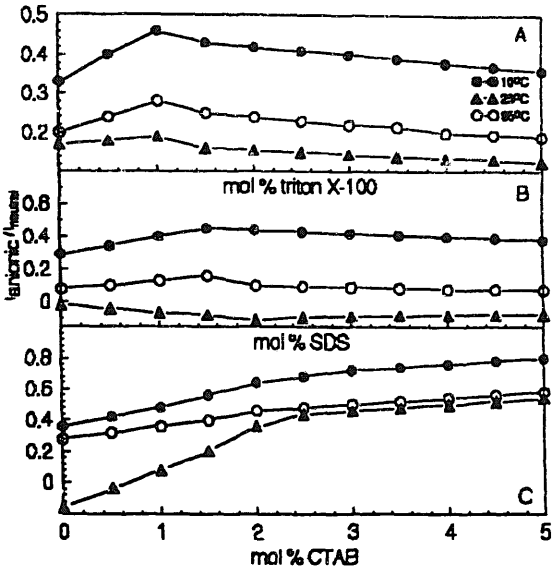


Fig. 2.

equilibrium is affected. Hence, it can serve as a potential probe for monitoring the changes in the properties of liposomes due to the binding of surfactants [17]. The neutral peak emission is monitored at 360 nm and the anionic peak at 478 nm (excitation at 300 nm). The variation in the ratio of the anionic to the neutral form emission intensity on the addition of different surfactants is given in Fig. 2.

In the presence of Triton X-100 (Fig. 2(A)) in liposome, the anionic to neutral peak intensity increases up to 1 mol%, and on further addition it decreases and reaches an almost constant value. A similar effect is observed on the addition of SDS (Fig. 2(B)), but the increase in the ESPT is observed up to 1.5 mol%. In the presence of CTAB over the entire concentration range, there is an increase in the ESPT (Fig. 2(C)). A similar effect is observed on the addition of different surfactants to DMPC liposomes in the solid gel phase, liquid crystalline phase and also near the phase transition temperature (T_c). The extent of the effect was found to be greater in the solid gel phase than in the fluid phases.

3.3. Changes in the microviscosity of liposome as studied from the anisotropy of DPH

The variation in the microviscosity of liposomes on the addition of different surfactants, as determined from the fluorescence polarization of DPH incorporated into DMPC liposomes, is shown in Fig. 3.

In the solid gel phase (Fig. 3(A)), the microviscosity ($\bar{\eta}$) increases in the presence of all the three surfactants for concentrations up to 1 mol% for triton X-100, 1.5 mol% for SDS and 5% for CTAB. A decrease in the microviscosity is observed beyond 1 mol% and 1.5 mol% for triton X-100 and SDS respectively, after which it reverts to more or less the original value. A similar effect is observed in the liquid crystalline phase on the addition of different surfactants (Fig. 3(C)). Near the phase transition temperature

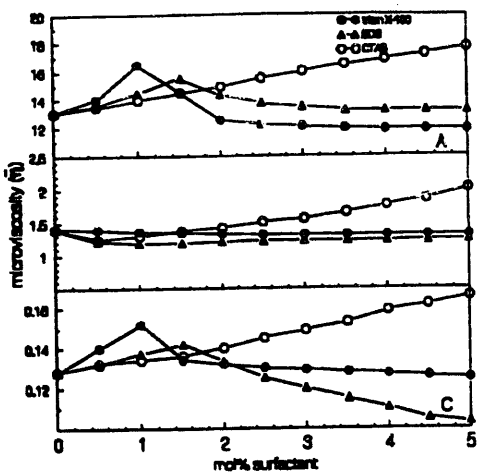


Fig. 3.

(Fig. 3(B)), the addition of triton X-100 as well as SDS has no appreciable effect on the microviscosity over the entire range of concentrations studied. A continual increase in the microviscosity was observed on the addition of CTAB.

3.4. Effect of surfactant on the thermotropic phase transitions

The effect of these surfactants in the thermotropic phase transition of DMPC liposome can be seen from a plot of $d(\ln \bar{\eta})/dT$ vs. T, as depicted in Figs. 4-6. On the addition of triton X-100 (Fig. 4) up to 1 mol%, lengthening of the endotherm was observed.

On further addition of triton X-100, the endotherm became shorter. No significant broadening of the endotherm was observed. A similar effect is observed in the case of SDS (Fig. 5), but the shortening is much more pronounced. However, on the addition of increasing amounts of CTAB (Fig. 6), the main endotherm is widened, shortened and finally disappears at 5 mol%.

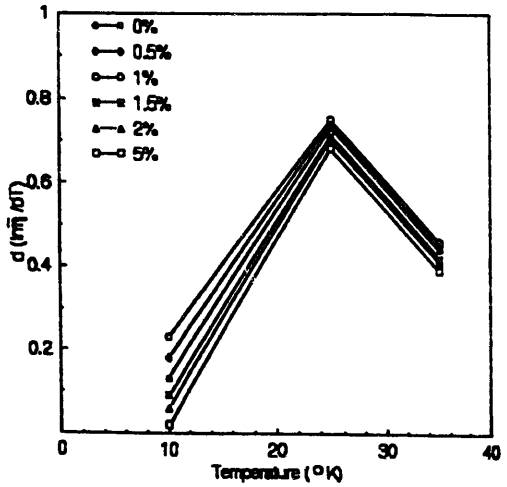


Fig. 4.

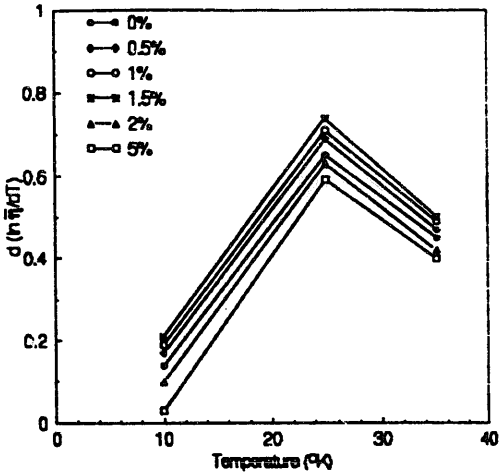


Fig. 5.

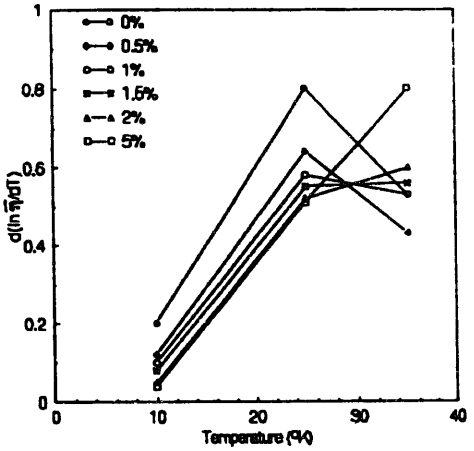


Fig. 6.

4. Discussion

A very sensitive measure of the perturbation of membrane morphology by surfactants is the change in fluidity and packing density of bilayers. This is studied from the change in microviscosity of DPH, which partitions into the inner hydrocarbon region of liposomes. Although the absolute changes observed here are small, the main interest is in the surfactant induced changes.

The increase in microviscosity observed at low concentrations of surfactants suggests an improvement in the packing of phospholipid molecules by the insertion of traces of these wedge shaped surfactants into the outer bilayer [14]. In the case of the neutral surfactant triton X-100, an increase in the packing density on the outer layer increases the surface area and decreases the accessibility to water. The small addition of negative charge due to insertion of SDS into the outer layer stabilizes the liposome by decreasing the van der Waals interaction between neutral vesicles, thus preventing fusion and aggregation [21b]. The reduction in the electrostatic charge of the headgroups as a result of the binding of positively charged CTAB causes the bilayer to condense, thus increasing the microvisosity [21c]. The concentrations up to which

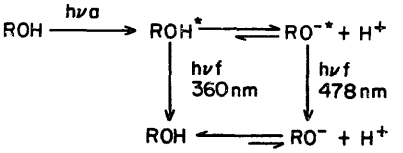
these effects are observed differ depending on the nature of the surfactant.

At higher concentrations of surfactants, in the case of triton X-100, because of a decrease in the lipid order parameter as observed from time resolved anisotropic measurements [14], there is a decrease in the microviscosity. The introduction of more negative charge by the addition of higher concentrations of SDS leads to electrostatic repulsion of the headgroups which pushes them apart, making the bilayer more permeable and thus decreasing the microviscosity [21c]. The kind of effect observed due to the binding of different surfactants in the different phase states of liposome can be explained on a similar basis. However, the effects are very much pronounced in the more ordered solid gel phase as it is very sensitive to any perturbations in the packing density. Moderate effects are observed in the fluid liquid crystalline phase as the membrane is loosely packed. The effects are minimal near the phase transition temperature because of the very high permeability of the vesicles and irregular packing due to coexistence of the phases [16].

The shortening of the endotherm of the thermotropic phase transition by all the surfactants is due to penetration of the additive into the bilayer, which prevents a portion of the lipid from participation in the phase transition by forming phospholipid–detergent mixed bilayer phases [23]. The extent to which it affects the phase transition is found to differ depending on the nature of the surfactant. In the case of triton X-100 and SDS, only moderate effects are observed in the thermotropic phase transitions, but CTAB produces much greater changes in the transition temperatures. This can be attributed to the increased positive charge on the membrane surface which alters the water of hydration, thus leading to the broadening and change in the phase transition temperature [24].

A striking resemblance (Figs. 1(A) and 3(A)) can be observed between the changes in the microviscosity as shown by DPH and those in the fluorescence of ANS on the addition of triton X-100. As ANS senses the changes in the properties of the membrane surface, the intensity changes on the addition of surfactant confirm that triton X-100, at low concentrations, is being inserted into the bilayer as described in a model suggested by Lasch et al. [14], thus increasing the packing order both on the surface and in the inner hydrocarbon region. Hence, the changes in intensity of ANS should be due to changes in the quantum yield of ANS. However, the intensity changes observed on the addition of SDS and CTAB do not resemble the changes in microvicosity. As these surfactants are charged, the changes caused should be more due to alterations in the charge density on the surface. Addition of SDS makes the surface of the vesicle more negative, thus reducing the number of ANS molecules, a negatively charged probe, binding to the surface. However, CTAB makes the surface positively charged, thus enabling more ANS molecules to bind to the surface, and an increase in the fluorescence emission of ANS is observed. Similar effects are observed over all three temperature ranges studied.

1-Naphthol, a ESPT probe, is distributed between two different sites in lipsomes, one on the surface and one in the inner hydrocarbon region, as given in the following scheme [17].



The relative population in the two sites varies depending on the permeability of the bilayer. Thus, the change in the relative intensity of the anionic to the neutral peak compares well with the change in microviscosity observed using DPH as a probe. When the membrane fluidity is decreased, 1-naphthol cannot penetrate into the inner hydrocarbon region, resulting in a increase of the population on the surface leading to increased anionic peak intensity. The effect is much more pronounced in the solid gel phase than in the liquid crystalline phase, because of greater order in the former. Triton X-100 and SDS produce very small changes in ESPT, whereas the changes produced by CTAB are appreciable. This can be attributed to the extent to which each surfactant is affecting the microviscosity.

5. Conclusion

The binding of detergents at low sublytic concentrations to phospholipid bilayers causes greater changes in their physical properties. The results obtained from the three conceptually different fluorescent probes ANS (a polarity probe), DPH (a polarization probe) and 1-naphthol (an excited state acid probe) all lead to the same conclusion: namely, the effect of the incorporation of different types of surfactants into the lipid bilayers depends on the nature and the concentration of the surfactant, on the position of the hydrophilic and hydrophobic parts of the molecule in the lipid bilayer and, more importantly, on the phase state of the liposomes. All the wedge shaped surfactants studied here lead to optimized packing up to a particular concentration. Ionic surfactants cause greater changes in the physical properties of liposomes than a neutral surfactant. The extent of these changes are greater in the more ordered solid gel phase than in the fluid liquid crystalline and disordered phases that exist near the phase transition temperature.

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