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Effect of sodium deoxycholate and sodium cholate on DPPC vesicles: A fluorescence anisotropy study with diphenylhexatriene

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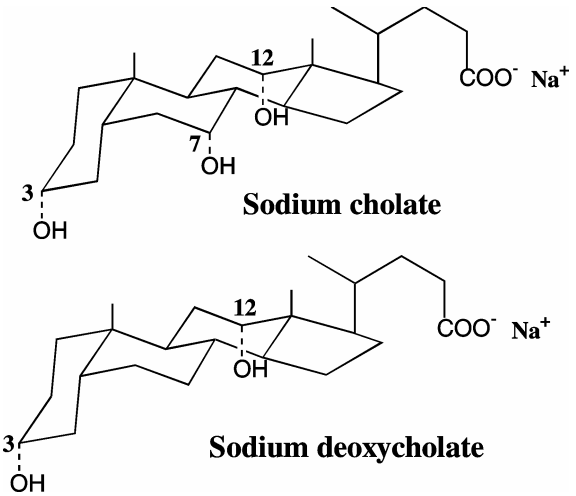
Abstract. Effects of two bile salts, namely sodium deoxycholate (NaDC) and sodium cholate (NaC), on DPPC small unilamellar vesicles have been investigated using the steady-state fluorescence anisotropy (r_{ss}) of diphenylhexatriene (DPH) as a tool. It was found that the variation of r_{ss} is sensitive enough to monitor different stages of interaction of bile salts with DPPC vesicles. NaDC induced significant changes in the membrane well below its CMC (6 mM). Even at 4 mM, which is still lower than the CMC, the phospholipids were completely solubilised by the NaDC micelles. The effect of NaC on DPPC vesicles, however, was much less significant, especially in the sub-micellar concentration regime. Being more hydrophilic NaC does not interact with the membrane efficiently. Complete solubilisation of phospholipids took place only when the concentration of NaC was above its CMC (16 mM). The experiments also showed that the bile salt-induced changes of vesicle structure were strongly dependent on the concentration of the bile salt and not on the molar ratio of lipid and bile salt.

Keywords. Bile salt; DPPC vesicle; fluorescence anisotropy; 1,6-diphenylhexatriene.

1. Introduction

Mixed lipid/detergent systems play an important role in the investigation of membrane properties and functions.[1,2] Studies of mixtures of micelle-forming bile salts and bilayer-forming phosphatidylcholines are of general interest not only for the understanding of the self-assembly of supramolecular aggregates,[3] but also for the design of new drug-delivery systems.[4,5] Progress in the development of highly effective drugs requires the optimisation of existing formulations and the creation of new delivery systems; hence the interest in bile salt/phospholipid mixed micellar systems. These aggregates aid in the solubilisation of poorly soluble drugs as well as in the inclusion of larger drugs. On addition of appropriate sublytic concentrations of bile salt to lipid bilayer, an expansion of the vesicles takes place and momentary defects are induced in liposomal membranes leading to enhanced permeability for molecules up to a size of 70,000 Daltons. This method is known as 'detergent-induced liposome loading' (DILL).[6] Although their biocompatibility and biodegradability make them safe and efficacious vehicles for medical applications, there still remain several problems to be overcome, one of such problems being the stability of lipid vesicles in blood and in the gastrointestinal tract.[7] The stability of liposomes in the gastrointestinal tract is largely affected by the bile salts; hence a proper understanding of the action of bile salts on liposomes is indispensable.

The interaction efficiency of bile salts with phospholipid vesicles depends on a number of factors such as chemical nature and concentration of the bile salt, molecular structure of the lipids, size,



Scheme 1. Molecular structures of sodium cholate (NaC) and sodium deoxycholate (NaDC).

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shape and state of the vesicles, buffer, pH, and temperature.[8,9] Hence, it is desirable to have a clear understanding of the interaction between bile salts and phospholipid vesicles under different experimental conditions and with different kinds of vesicles.

The objective of the present work was to investigate the effect of two bile salts dihydroxy sodium deoxycholate (NaDC) and trihydroxy sodium cholate (NaC) (scheme 1) on dipalmitoylphosphatidylcholine (DPPC) small unilamellar vesicles (SUVs), employing the fluorescence anisotropy of 1,6-diphenylhexatriene (DPH) as a tool.

2. Experimental

2.1 *Materials*

Dipalmitoylphosphatidylcholine (DPPC) was purchased from the Sigma Chemical Company (USA) and was used as such. Sodium deoxycholate (NaDC) and sodium cholate (NaC) were obtained from SRL India Ltd. and were recrystallized from hot ethanol. 1,6-diphenylhexatriene (DPH) was purchased from the Sigma Chemical Company (USA) and was used as received. Water, distilled twice from alkaline permanganate solution, was used for the experiments.

2.2 *DPPC vesicle preparation*

For the present work small unilamellar vesicles in the size range 30–50 nm were used. The vesicles were prepared by ethanol injection method,[10] where an ethanolic solution of the lipid was injected rapidly with the help of a fine needle into water maintained at 50°C (optimised condition). The volume of ethanol injected is always less than 1% v/v in order to avoid any damage to the liposome by ethanol.

2.3 *Labelling of vesicles*

Labelling of the vesicles was achieved by adding a measured amount of DPH to the lipid solution before preparation of the liposome, in a molar ratio yielding the desired final lipid/probe ratio, such that DPH is directly incorporated into the membrane during its formation. For all the experiments, a control solution containing the same concentration of lipid in absence of probe was used as blank. The partition coefficient value (K_p) of DPH in DPPC SUVs is calculated using the equation

$$F = F_0 L/\{(55{\cdot}6/K_p) + L\},$$

where F is the fluorescence intensity at 428 nm at lipid concentration L, F_0 is the maximum fluorescence resulting from total probe incorporation into membrane.[11] The K_p value is found to be 2×10^6 in the liquid crystalline phase (50°C). From this value the lipid to probe ratio is calculated to be ≈600 by using [DPH] = 5×10^{-7} M and [DPPC] = 3×10^{-4} M and this ratio was maintained for further work.

2.4 *Incorporation of bile salts*

Stock solutions of NaDC and NaC were prepared in water. Fresh solutions of bile salt were prepared every time to avoid the problem of aging. The experimental solutions were prepared by adding the desired volume of bile salt stock to the preformed DPPC vesicles and the solution was equilibrated for at least two hours before experiments.

2.5 *Fluorescence measurements*

Fluorescence measurements were carried out with Jobin-Yvon fluorolog II spectrofluorimeter. Excitation and emission spectra were recorded with 5/5 nm slit widths. For temperature dependence experiments the temperature was controlled by circulating water through a jacketted cuvette holder from a refrigerated bath (Insref Ultra Cryostat, India). Temperature was also checked inside the cuvette before and after the experiments; the variation was negligible. The steady-state fluorescence anisotropy (r_{ss}) values were obtained using the expression

$$r_{ss} = (I_{\parallel} - GI_{\perp})/(I_{\parallel} + 2GI_{\perp}),$$

where $I_{\parallel}$ and $I_{\perp}$ are fluorescence intensities when the emission polarizer is parallel and perpendicular, respectively, to the direction of polarization of the excitation beam, and G is the factor that corrects for unequal transmission by the diffraction gratings of the instrument for vertically and horizontally polarized light.

3. Results and discussion

DPH is one of the most popular fluorescence anisotropy probes for bilayer membranes.[12–14] It is a rod-like molecule, whose absorption and emission

dipoles are collinear and lie along the principal molecular axis, which is perpendicular to the membrane plane. The probe is located deep within the hydrophobic core region of the phospholipid bilayer.[14] Thus any modification in the movement of the chains or packing is sensitively reflected in the fluorescence anisotropy of DPH. Both fluorescence intensity and fluorescence anisotropy of DPH are found to show very good response to the phase transition in phospholipid vesicles. The anisotropy shows a sudden drop at 42°C, the phase transition temperature of DPPC. Hence, it was chosen to monitor the effect of bile salt on phospholipid vesicles. The critical micelle concentrations (CMC) of the two bile salts NaDC and NaC in water (25°C) were determined by using the fluorescent dye method employing DPH fluorescence intensity and anisotropy as tools.[15,16] The CMCs thus found are 6 mM for NaDC and 16 mM for NaC.

3.1 *Effect of low concentrations of NaDC and NaC on DPPC SUVs*

Figures 1a and b show the variation of fluorescence anisotropy of DPH with temperature in DPPC SUVs on addition of various concentrations of NaDC and NaC, respectively. The concentration of bile salt was varied between 0·1 and 1 mM, keeping the DPPC concentration constant at 0·3 mM. Plots for a few intermediate concentrations have been removed from the figure for clarity.

There is no appreciable change in the anisotropy profiles of DPH observed in DPPC SUVs in presence of NaC in this concentration range, which clearly indicates that NaC, when present at low concentration, does not have significant effect on the phase behaviour of DPPC SUVs. However, unlike NaC, the effect of NaDC on DPPC SUVs is remarkable even at these low concentration ranges. Variation of fluorescence anisotropy shows behaviour similar to that in pure DPPC SUVs up to 0·55 mM of NaDC, i.e. the anisotropy is high at lower temperature and shows a drop at the phase transition and then levels off on further increase in temperature. However, there is an almost linear decrease in phase-transition temperature in the concentration range of 0·1 mM to 0·55 mM of NaDC; below 0·1 mM of NaDC the phase-transition temperature remains practically the same as that of pure DPPC SUVs (42°C). The transition temperature decreases from 42°C in pure DPPC to 35°C by 0·55 mM of NaDC (figure 2). This indicates that up to 0·55 mM of NaDC, incorporation of deoxycholate into the DPPC bilayer causes a disturbance in the interactions among the chains resulting in a decrease in the temperature of the gel to liquid crystal transition. Thus, in the above concentration range (0·1–0·55 mM), NaDC has a fluidising effect on DPPC SUVs; the bilayer structure of bile salt incorporated vesicles is still intact showing distinct phase behaviour.

In the concentration window 0·6 to 1·0 mM of NaDC, the anisotropy shows variation similar to that of a pure DPPC bilayer below the phase transition temperature, but above this temperature it increases with increasing temperature, which is similar to that in pure NaDC micelles.[16] It is known in the literature that the partition coefficient of NaDC depends on the physical state of the lipid membrane.[17] Thus, in the present case it can be argued that as the temperature approaches the phase transition temperature more fluid regions in the bilayers are likely to arise, which in turn increases the permeability of the

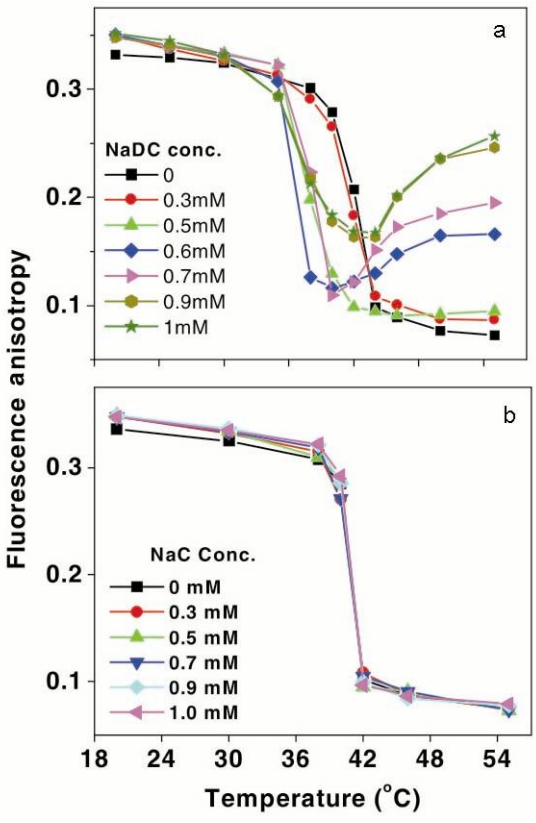


Figure 1. Response of DPH fluorescence anisotropy to the changes induced by (**a**) NaDC and (**b**) NaC (0–1 mM) in 0·3 mM of DPPC SUVs (λ_{ex} = 360 nm, λ_{em} = 428 nm).

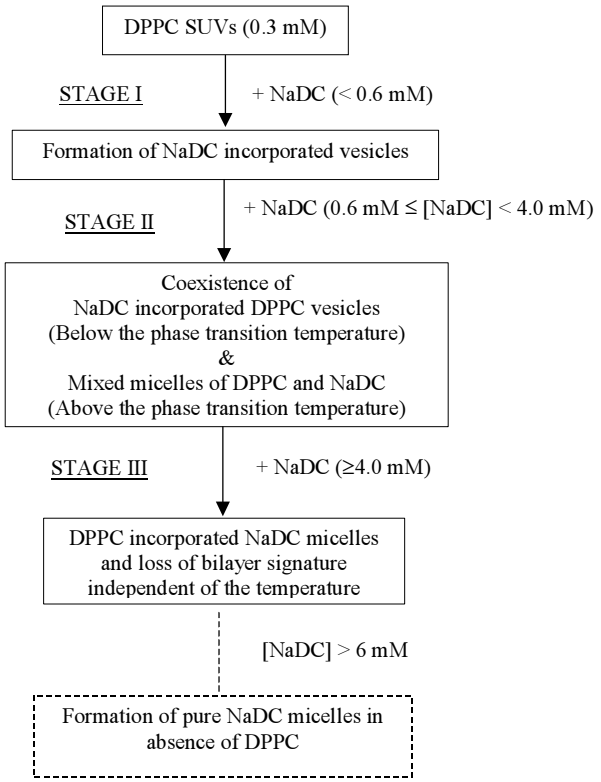
membrane towards NaDC so as to facilitate the formation of mixed micelles. Therefore, in the above concentration range of NaDC (0·6–1 mM) there is coexistence of bile salt incorporated bilayers (at low temperature, i.e. below the phase transition temperature) and lipid-rich mixed micelles (at high temperature).

3.2 *Effect of high concentrations of NaDC on DPPC SUVs*

Figure 3 shows the fluorescence anisotropy profiles of DPH with temperature in DPPC SUVs in the presence of NaDC in the concentration range of 1·0 mM $\leqslant$ [NaDC] $\leqslant$ 6·0 mM. As the concentration of NaDC increases from 1·0 to 3·0 mM, the micellar character becomes prominent; however there still exists the signature of bilayer phase transition at lower temperature. By 4·0 mM of NaDC the phase transition is completely abolished and the anisotropy profiles exactly resemble those of pure NaDC micelles. This indicates that above 4·0 mM of NaDC, which is still lower than its CMC in water (6 mM), the bilayer signature is completely lost and there is existence of micelles suggesting the solubilisation of phospholipids. There are literature reports on decrease in critical micelle concentration of bile salt on addition of phosphatidylcholine.[18,19]

The solubilisation of lipid vesicles by detergents such as Triton-X is known to proceed through four stages: Stage I: mixed vesicle formation, stage II: coexistence of mixed vesicles and mixed bilayer sheets, stage III: transformation of mixed bilayers to lipid-rich micelles and stage IV: solubilisation of lipid in the detergent micelles.[20] From our experimental data, a similar model shows the solubilisation of DPPC SUVs by NaDC. Thus, under experimental conditions, 0·3 mM of DPPC SUVs, the different stages can be depicted as follows: (i) at [NaDC] < 0·6 mM, NaDC partitions into the bilayer and alters the chain order resulting in linear decrease in the phase transition temperature with NaDC concentration; (ii) at 0·6 mM $\leq$ [NaDC] < 4·0 mM, there is coexistence of mixed vesicles and micelles, below the phase transition the bilayer signature is observed and near the phase transition temperature the permeability of the bilayer for NaDC increases, thus more of NaDC get associated with the bilayer, converting them to mixed micelles; (iii) at [NaDC] $\geq$ 4·0 mM, complete solubilisation of DPPC lipids in NaDC micelles takes place and there is no signature of bilayer observed.

This can be presented in a flow diagram as below:



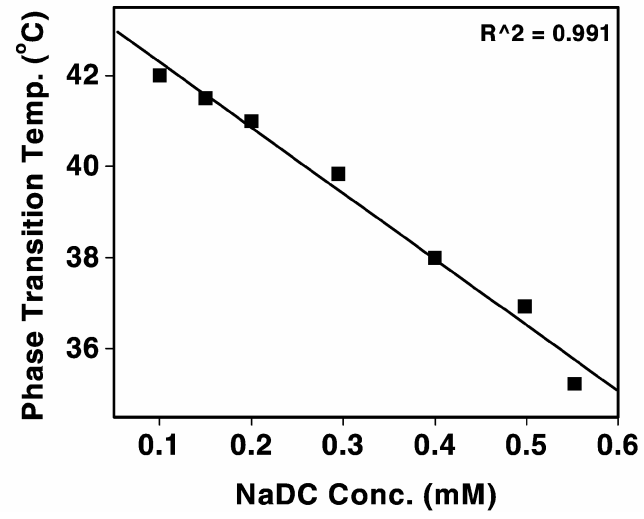


Figure 2. Variation of the main phase transition temperature of DPPC SUVs with NaDC concentration.

3.3 *Effect of high concentrations of NaC on DPPC SUVs*

Figure 4 shows temperature-dependent anisotropy profiles of DPH in DPPC SUVs indicating the effect

of NaC on DPPC SUVs in the higher concentration range. NaC concentration is varied from 4–20 mM. The bilayer behaviour is retained even up to a concentration of 14 mM of NaC. However, the phase transition temperature diminishes with increase in NaC concentration; it drops from 42°C in pure DPPC to 32°C in presence of 14 mM of NaC. Above 16 mM of NaC the anisotropy profile shows similar trend as that of pure NaC micelles in the higher temperature regime. In case of NaC, the complete solubilisation of phospholipid vesicles takes place only when the concentration of NaC is above its CMC.

The efficiency of a bile salt towards membrane perturbation is found to be dependent on its nature. For efficient perturbance to occur at a concentration prior to the critical micelle concentration of the bile salt, it is expected that the affinity of the bile salt for the membrane should be high as compared to the affinity of the bile salt for itself, which is clearly the case for the lipophilic deoxycholate. NaC being more hydrophilic does not interact with the membrane efficiently and hence its solubilising capacity towards phospholipid vesicles is less.

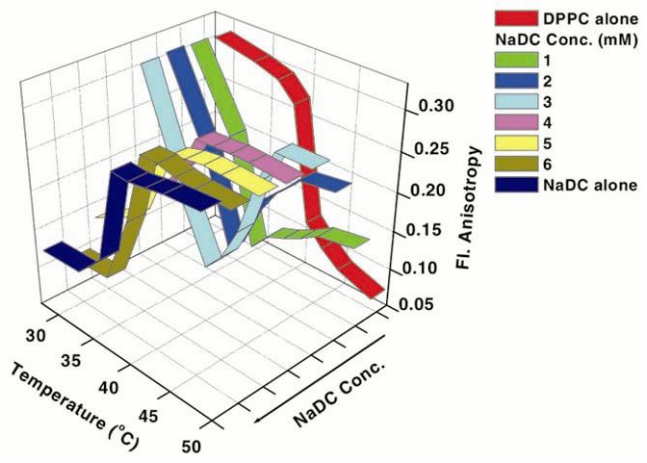


Figure 3. 3D plots showing variation of steady-state fluorescence anisotropy of DPH in DPPC SUVs as a function of temperature. [DPPC] = 0·3 mM, [NaDC] = 1·0–6·0 mM (λ_{ex} = 360 nm, λ_{em} = 428 nm). The variation of fluorescence anisotropy in pure NaDC micelles is also given for comparison.

3.4 *Experiments with different concentrations of DPPC SUVs*

It was found that the first appearance of mixed micelles occurs at 0·6 mM of NaDC for 0·3 mM of DPPC SUVs, which comes out to be a ratio of 1 : 2 of lipid to NaDC. In order to examine whether this ratio of lipid to NaDC is important for mixed micelle formation, experiments were carried out with different concentrations of DPPC SUVs and NaDC maintaining their ratio at 1 : 2.

Figure 5 shows the temperature-dependent fluorescence anisotropy profiles of DPH at different concentrations of DPPC SUVs and NaDC mixed in a fixed ratio of 1 : 2. It is clear from the figure that although the ratio of DPPC to NaDC is kept con-

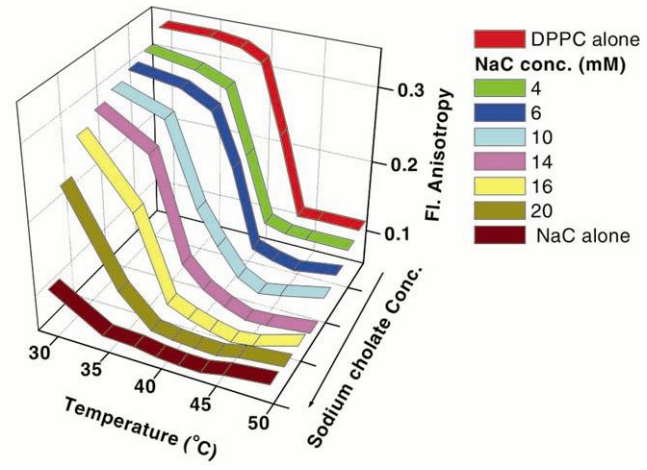


Figure 4. Temperature-dependent steady-state fluorescence anisotropy profiles of DPH in DPPC SUVs in presence of NaC at higher concentration range. [DPPC] = 0·3 mM. [NaC] = 4·0–20 mM (λ_{ex} = 360 nm, λ_{em} = 428 nm). The temperature dependence of anisotropy in pure NaC micelles is also given for comparison.

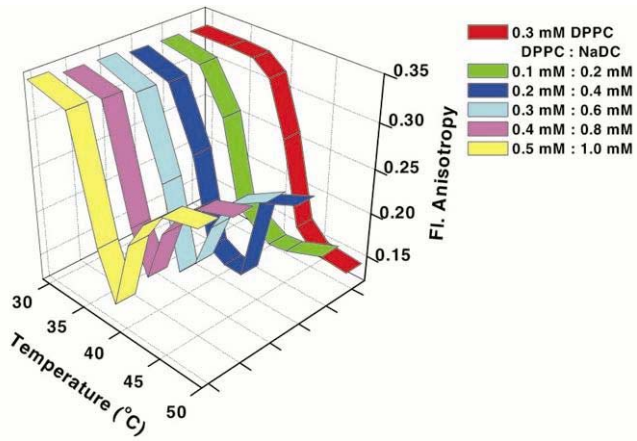


Figure 5. Temperature-dependent steady-state fluorescence anisotropy profiles of DPH in mixture of DPPC SUVs and NaDC at a ratio of 1 : 2. [DPPC] = 0·1–0·5 mM and [NaDC] = 0·2–1·0 mM (λ_{ex} = 360 nm, λ_{em} = 428 nm).

stant, the anisotropy profiles differ from each other. This indicates that the formation of mixed micelles of phospholipid vesicles and bile salts is strongly dependent on the absolute concentrations of lipid and bile salt and not just on their molar ratio. This is in agreement with earlier literature reports that the effect of surfactant on bilayer depends on the "effective" molar ratio of detergent/phospholipid in the bilayer and not on the "total" molar ratio[17].

4. Conclusions

The present study provides an insight into the mechanism of interaction of bile salts with small unilamellar vesicles. Based on the experimental findings it is possible to propose the concentration ranges involved with different stages of changes in the solubilisation of DPPC SUVs by NaDC. The experiments showed that the concentration of a bile salt needed to bring about a certain change in the vesicle is strongly dependent on the absolute concentrations of both phospholipid and a bile salt and not on their molar ratio. As compared to NaDC, the effect of NaC on DPPC SUVs is found to be subtle. NaC when added at a concentration above its CMC (16 mM) is found to solubilise the vesicles.

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