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# Fluorescence (Fluidity/Hydration) and Calorimetric Studies of Interactions of Bile Acid−Drug Conjugates with Model Membranes

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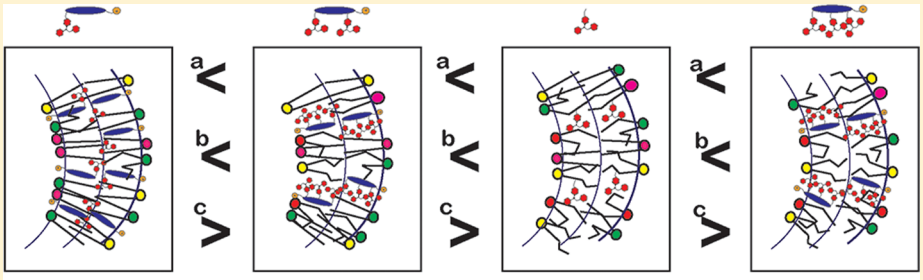


**ABSTRACT:** We have studied the interactions of three bile acid−tamoxifen conjugates, lithocholic acid−tamoxifen (LA-Tam$_1$-Am), deoxycholic acid−tamoxifen (DCA-Tam$_2$-Am), and cholic acid−tamoxifen (CA-Tam$_3$-Am), possessing 1−3 tamoxifen molecules having an amine headgroup with model DPPC membranes and compared with *N*-desmethylated tamoxifen (TamNHMe) using DPH based fluorescence anisotropy, Prodan based hydration, and differential scanning calorimetry studies. DPH based anisotropy studies showed that bile acid−tamoxifen conjugates increase membrane fluidity, which strongly depends on the number of tamoxifen molecules conjugated to bile acid and the percentage of doping of bile acid−tamoxifen conjugates in the DPPC membranes. The order of membrane fluidity of the coliposomes from bile acid−tamoxifen conjugates and DPPC lipids in gel phase was found to be CA-Tam$_3$-Am > DCA-Tam$_2$-Am > LA-Tam$_1$-Am > TamNHMe. Incorporation of bile acid−tamoxifen conjugates showed an unusual complex behavior of membrane hydration, as evident from Prodan based hydration studies. Temperature dependent study showed incorporation of LA-Tam$_1$-Am and DCA-Tam$_2$-Am conjugates decreases membrane hydration with an increase in temperature up to the phase transition temperature ($T_m$). Differential scanning calorimetry studies showed a decrease in phase transition temperature ($T_m$) upon an increase in the percentage of doping of TamNHMe and CA-Tam$_3$-Am, whereas LA-Tam$_1$-Am and DCA-Tam$_2$-Am do not cause a major change in the phase transition temperature ($T_m$) of DPPC liposomes. These studies showed the differential behavior of bile acid−tamoxifen conjugates regulating the membrane fluidity, hydration, and phase transition of model membranes depending upon the percentage of doping and tamoxifen conjugation to bile acids.

## INTRODUCTION

Cell membrane plays an important role for cellular protection that also regulates transport of ions, nutrients, and drug molecules.[1] Interactions of drugs with cellular membranes influence therapeutic effects of these drugs that further helps in design and discovery of new therapeutics.[2] Drug−membrane interactions can be used to predict pharmacokinetic properties of drugs such as transport, biodistribution, accumulation, efficacy, and mechanism of transport depending upon physical and chemical properties of drugs like size, hydrophobicity, and hydrophilicity etc.[3] Interactions of drugs with biological membranes modulates drug activity and toxicity as drug−membrane interactions strongly affect the structure and properties of biological membranes. Drug−membrane interactions can change many biophysical features of membranes like the conformation of acyl chains, phase transition, membrane thickness, hydration of headgroups, membrane potential, and membrane fusion properties.[4]

Drug or drug delivery vehicles can interact with lipid membranes using three different types of interactions: (1) interactions with headgroups of lipids; (2) interactions with hydrophobic alkyl chains; (3) interactions with headgroups and hydrophobic chains influencing fluidity and packing of lipid membranes.[5] The dynamic nature of cellular membranes makes it challenging to understand the interactions of drugs or drug delivery vehicles with cell membranes. Model membrane systems like bilayers,[6] surface monolayers,[7] liposomes,[8] etc. and their interactions with drugs and drug delivery systems play a critical role in understanding the cellular uptake and

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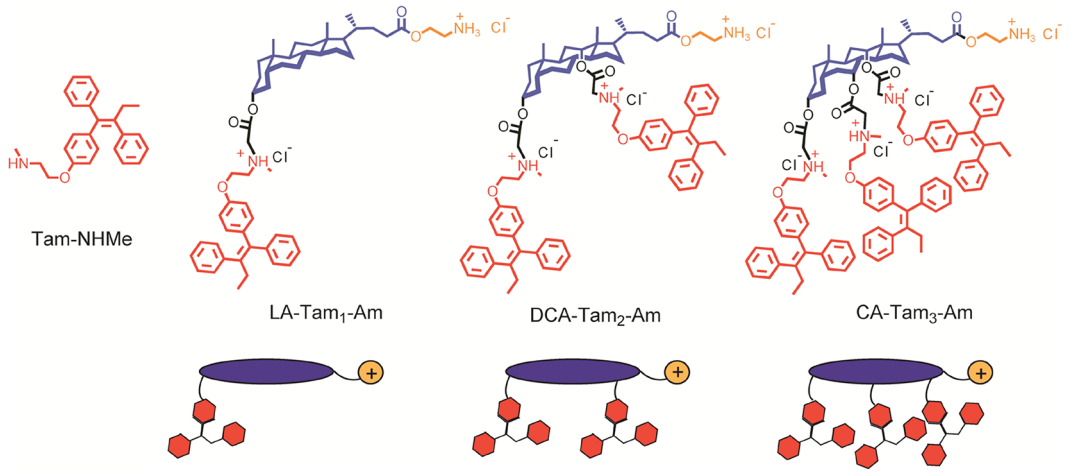


**Figure 1.** Molecular structures of *N*-desmethylated tamoxifen (TamNHMe) and different bile acid−tamoxifen conjugates (LA-$Tam_1$-Am, DCA-$Tam_2$-Am, CA-$Tam_3$-Am) used for the study.

predicting toxicity of drugs, thereby helping in developing new drug molecules and optimizing drug delivery systems. Different biophysical techniques[9] have been used to study the interactions of drugs like antibiotics,[10] antihypertensive,[11] antifungal,[12] antipsychotic,[13] and anticancer drugs with model membrane systems.[14] Lucio et al. studied binding of non-steroidal anti-inflammatory drugs with DPPC membranes and studied their effect on phase transition and phase properties.[15] Interactions of albumin with DPPC membranes and effect of PEGylated lipids have been studied by Franses and co-workers.[16] Gzyl-Malcher et al. studied the effect of organic and nonorganic ions like indolilo-3-acetic acid and selenite ions on morphology of mixed DPPC/DPTAP monolayers.[17]

Tamoxifen is a nonsteroidal antiestrogen that has been used for breast cancer therapy.[18] Anticancer effects of tamoxifen have also been reported in estrogen negative cell lines apart from classical esterogen-receptor model. Tamoxifen gets incorporated into biomembranes and also inhibits enzymes involved in cell proliferation by interacting with phospholipids.[19] Fluorescence anisotropy studies have shown that tamoxifen also decreases membrane fluidity in ER-positive and ER-negative breast cancer cell lines. Interactions of tamoxifen with DPPC and DMPC membranes showed fluidizing effect and decrease in main phase transition temperature ($T_m$).[20]

Bile acids are natural surfactants that possess a convex side of a hydrophobic steroidal backbone and a hydrophilic side having hydroxyl groups on the concave side, making these molecules as facial amphiphiles.[21] Bile acids and their derivatives have extensively been studied for many biological processes like solubilization of lipids, cholesterol, bilirubin, lecithin, and fat-soluble vitamins.[22] Interactions of bile salts and phospholipid membranes occur during liposomal drug delivery in hepatobilary systems. Bile acid−drug conjugates have recently been explored for anticancer activities and efficient delivery of drugs.[23] Bile salts are known to bind efficiently with membranes and also enhance rate of intervesicular phospholipid transfer process. Mishra and co-workers have shown interactions of bile salts with DPPC membranes at submicellar concentrations of bile salts that induce hydration of DPPC and DMPC unilamellar vesicles.[24] Winter et al. have studied the effect of different steroidal molecules like cholesterol, ergosterol, *β*-sitosterol, *trans*-7-dehydrocholesterol, stigmasterol, and lanosterol on DPPC membranes.[25] Therefore, the study of interactions of steroidal bile acid based lipid−drug conjugates with model DPPC membranes would help us in understanding the future design of bile acid based lipid−drug conjugates for anticancer therapy applications.

We have synthesized a series of bile acid−tamoxifen conjugates having an amine headgroup for breast cancer therapy. To understand the mechanism of interactions of these bile acid−drug conjugates with membranes, we performed interactions of an amine headgroup containing bile acid−tamoxifen conjugates (LA-$Tam_1$-Am, DCA-$Tam_2$-Am, CA-$Tam_3$-Am) (Figure 1) with model DPPC membranes using fluorescence anisotropy, Prodan based hydration, and differential scanning calorimetry studies and compared with *N*-desmethylated tamoxifen (TamNHMe). Fluorescence anisotropy studies showed that incorporation of bile acid−tamoxifen conjugates fluidize DPPC membranes in their gel phase below the phase transition temperature, and fluidity in general increases with an increase in the percentage of incorporation of conjugate. Prodan based hydration studies showed a decrease in surface hydration upon incorporation of LA-$Tam_1$-Am and DCA-$Tam_2$-Am up to the phase transition temperature ($T_m$), whereas CA-$Tam_3$-Am causes a more hydrated surface depending upon temperature. Phase transition temperature ($T_m$) studies using differential scanning calorimetry showed that TamNHMe and CA-$Tam_3$-Am fluidize DPPC membranes and lower the phase transition temperature as compared to LA-$Tam_1$-Am and DCA-$Tam_2$-Am.

## EXPERIMENTAL SECTION

**Materials.** All the chemicals used in the studies are of ACS grade. 1,6-Diphenylhexa-1,3,5-triene (DPH) and Prodan were obtained from Sigma-Aldrich, and dipalmitoyl phosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids.

**Liposome Formation.** [26] The desired amounts of DPPC and TamNHMe or bile acid−tamoxifen conjugate were taken in round-bottom wheaton glass vials in chloroform. Thin films were made under dry argon gas and were dried under vacuum for 6 h. Lipid films were hydrated with Milli Q water for overnight. Hydrated films were then processed for 4−5 freeze thaw cycles from 70 to 4 °C with intermittent vortexing. Multilamellar vesicles were then sonicated at 70 °C for 15 min to get unilamellar vesicles.


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**Fluorescence Anisotropy Studies.** [27] Fluorescence anisotropy studies were performed on unilamellar DPPC vesicles incorporated with different percentages of TamNHMe, and bile acid−tamoxifen conjugates. DPPC (0.5 mg) and the appropriate amount of TamNHMe or bile acid−tamoxifen conjugate along with DPH to make the final DPPC:DPH ratio 100:1 were taken in chloroform solution in wheaton vials. Thin films of mixed DPPC solution were prepared under a steady stream of dry argon gas. Thin films were further dried for 6 h under high vacuum. Thin films were then hydrated with Milli Q water to make a final DPPC concentration of 1 mM, and hydration was continued for at least 6 h at 4 °C. Hydrated lipid films were then freeze thawed for five cycles for optimal hydration by heating at 70 °C and cooling at 4 °C with intermittent vortexing. Lipid suspensions were then sonicated for 15 min at 70 °C to make unilamellar vesicles. Steady state anisotropy measurements were done in a 96-well plate using the fluorescence anisotropy protocol in a Molecular devices M5 instrument with $\lambda_{ex}$ at 350 nm and $\lambda_{em}$ at 452 nm. The effect of temperature on anisotropy was studied by measuring the fluorescence from 23 to 55 °C. Samples were equilibrated for at least 5 min after each temperature change. Steady state fluorescence anisotropy ($r_s$) was then calculated using the following equation

$$r_s = (I_{\parallel} - GI_{\perp})/(I_{\parallel} + 2GI_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are the emission intensity excited with parallel-polarized light and measured with the emission polarizer oriented in a parallel or perpendicular direction to the plane of excitation, respectively. $G$ is an instrument specific factor calculated to correct instrument polarization, which is equal to $I_{\perp\parallel}$ /$I_{\perp\perp}$ and obtained by measuring the parallel and perpendicular polarized emission intensities after excitation with perpendicularly polarized light. Sigmoidal curve fitting of experimental points were performed to deduce the phase transition temperature using Origin software. The phase transition temperature due to gel-to-liquid crystalline transition was calculated from midpoints of breaks from temperature dependent anisotropy values.

**Prodan Based Hydration Studies.** [28] Unilamellar mixed vesicles comprising DPPC and TamNHMe or desired bile acid−tamoxifen conjugates along with Prodan were prepared in a similar way as described for anisotropy studies. The generalized polarization experiments were performed in a 96-well plate in a Molecular Devices M5 instrument. The fluorescence experiments were performed using $\lambda_{ex}$ of 350 nm and end point emissions were recorded at $\lambda_{em}$ of 440 and 490 nm. The effect of temperature on polarization was studied by measuring the fluorescence from 23 to 55 °C. Samples were equilibrated for at least 5 min after each temperature change. The generalized polarization (GP) was calculated using the equation $GP = (I_{440} - I_{490})/(I_{440} + I_{490})$.

**Differential Scanning Calorimetry.** [29] DPPC liposomes and coliposomes of DPPC with TamNHMe or bile acid−tamoxifen conjugates were prepared as mentioned above. Differential scanning calorimetry studies were performed on a Nano DSC instrument, TA Instruments. All reference and sample solutions were degassed prior to being placed in the DSC to minimize the possibility of gas bubble formation during the run. We have pulled a vacuum of 15−25 in. Hg on solutions for a period of 10−15 min to degas a sample. Baseline runs were performed by filling both the sample cell and reference cells with Milli Q water. Liposomal samples were run by filling the sample cell with liposomal solution and the reference cell with Milli Q water. The DSC measurements were carried out in the temperature range 20−60 °C with heating and cooling scan rates maintained at 0.5 °C/min for all experiments. The DSC thermograms for liposomal vesicular suspensions of DPPC and coliposomes were obtained by subtracting the respective baseline thermogram from the sample thermogram using software NanoAnalyze data analysis provided by the manufacturer. The peak position in the plot of "excess heat capacity" vs temperature on the heating scan was taken as the solid-like gel-to-fluid liquid-crystalline phase transition temperature for each membranous suspension. The maximum point of excess heat capacity ($C_p^{max}$), calorimetric enthalpies ($\Delta H_c$), entropies ($\Delta S$) and full width at half-maximum (fwhm) were also computed using the same software as reported.

The size of cooperativity unit (CU) for phase transition of each lipid was determined using the formula[29]

$$CU = \Delta H_{vH}/\Delta H_c$$

We have calculated the van't Hoff enthalpy using calorimetric enthalpy. $T_m$ is the absolute phase transition temperature of drug doped sample, and $C_p^{max}$ is the maximum point of excess heat capacity in the main transition peak. First, the $C_p$ vs $T$ output scan from the calorimeter is integrated to form a plot of the enthalpy for the phase transition, $\Delta H_c$. The maximum of the $C_p$ vs $T$ curve is $C_p^{max}$. The van't Hoff enthalpy is expressed by[30]

$$\Delta H_{vH} = (X)(4RT_m^2C_p^{max})/(\Delta H_c) \approx (X)(6.9T_m^2/\Delta T_{1/2})$$

As $\Delta H_{vH}$ depends on the mole fraction of doped drug, we incorporated the mole fraction component ($X$) in the formula and calculated the van't Hoff enthalpy.

## RESULTS AND DISCUSSION

**Fluorescence Anisotropy Studies.** Membrane fluidity is a critical factor for the activity of liposomal drug delivery systems and for interactions of drugs with model membranes. The influence of drugs and drug delivery vehicles on membrane fluidity was studied using fluorescence anisotropy studies using DPH as a probe. DPH sits between phospholipid hydrophobic tails and senses the hydrophobic environment of liposomes. In the gel phase at room temperature, dynamic rotational motion of DPH is restricted and we would observe a high anisotropy. With an increase in temperature, liposomes make a transit from gel to liquid crystalline phase, where rotational motion of DPH is not restricted and lower anisotropy values are observed. Thus DPH polarization reports the rotational motion of probe that strongly depends on fluidic ordering of acyl chain of phospholipids. We first investigated for any possible interference on fluorescence emission of probes by TamNHMe and bile acid−tamoxifen conjugates as these conjugates have large aromatic moieties. We used 1% DPH solution in chloroform (nonpolar) as DPH senses in hydrophobic regions of lipids, and recorded fluorescence spectra on incubation with the highest concentrations (50%) of lipid−drug conjugates that are used in liposomal samples. We have not observed any change in the fluorescence properties of DPH, thereby confirming that even DCA-Tam$_2$-Am and CA-Tam$_3$-Am do not interact with DPH in monomeric state.[20]

Incorporation of *N*-desmethylated tamoxifen (TamNHMe) in DPPC membranes causes a little decrease in the anisotropy of DPPC membranes in gel phase (Figure 2a), indicating a little


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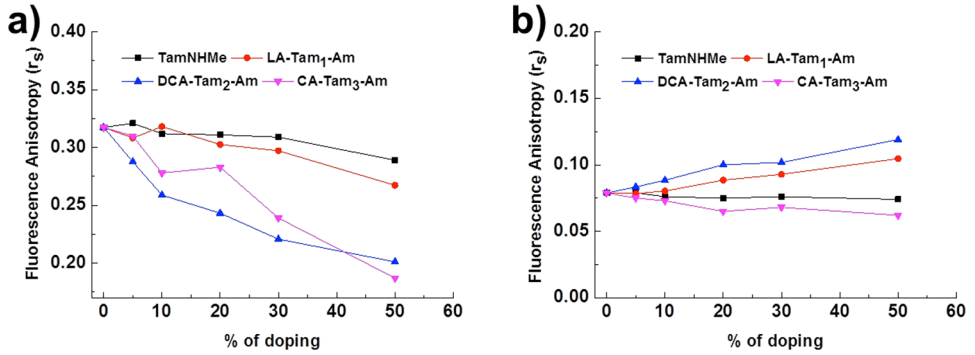


**Figure 2.** Changes in DPH anisotropy (membrane rigidity/fluidity) of DPPC membranes in the gel phase (a) at 25 °C and liquid crystalline phase (b) at 55 °C upon incorporation of TamNHMe and bile acid−tamoxifen conjugates LA-Tam$_1$-Am, DCA-Tam$_2$-Am, and CA-Tam$_3$-Am.

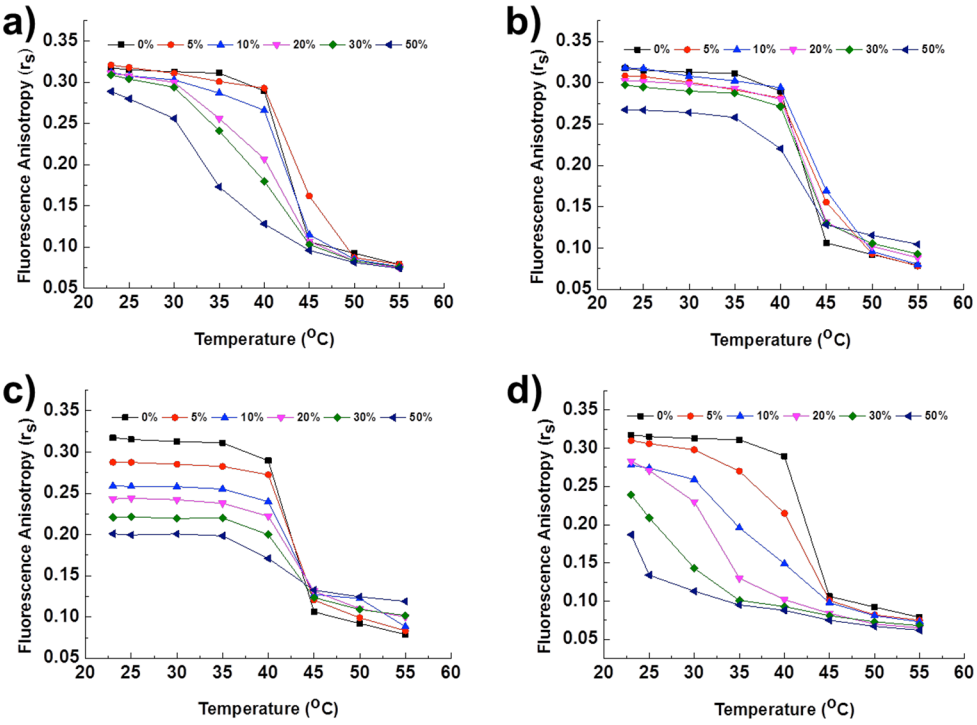


**Figure 3.** Effect of temperature on the DPH anisotropy (membrane rigidity/fluidity) of DPPC membranes upon doping of different percentages of TamNHMe (a) and bile acid−tamoxifen conjugates LA-Tam$_1$-Am (b), DCA-Tam$_2$-Am (c), and CA-Tam$_3$-Am (d).

increase in membrane fluidity upon incorporation of even 50% of *N*-desmethylated tamoxifen (TamNHMe). Bile acid−tamoxifen conjugates upon incorporation in DPPC membranes increases membrane fluidity to a greater extent as compared to *N*-desmethylated tamoxifen (TamNHMe). Among three bile acid−tamoxifen conjugates, DCA-Tam$_2$-Am and CA-Tam$_3$-Am cause more fluidity in DPPC membranes as compared to LA-Tam$_1$-Am. DCA-Tam$_2$-Am and CA-Tam$_3$-Am inhibit gel phase interactions of DPPC lipids, causing more fluidity as more tamoxifen molecules are incorporated on these molecules. In the liquid crystalline phase (LC) of DPPC membranes (Figure 2b), TamNHMe and CA-Tam$_3$-Am do not cause any noticeable change in membrane fluidity, whereas LA-Tam$_1$-Am and DCA-Tam$_2$-Am decrease membrane fluidity in the LC phase of DPPC membranes. LC membrane fluidity decreases with increase in % of doping for DCA-Tam$_2$-Am as compared to LA-Tam$_1$-Am.

In gel or solid-like phases of membranes, fatty acid chains of DPPC are arranged in staggered *s*-trans conformations, and van der Waals interactions between hydrophobic chains of polymethylene interact strongly to stabilize gel phase of membranes. Membranes make a transition from gel solid-like phase to fluid LC phase with an increase in temperature, where polymethylene chains of the hydrophobic tails are randomly arranged and methylene groups are in *s*-gauche conformations that disrupt the packing of the lipid molecules, giving a fluid-like interior of membranes.

Temperature based studies showed that doping of TamNHMe influences the phase transition temperature ($T_m$) of DPPC liposomes as we observed a decrease in $T_m$ upon an increase in doping of TamNHMe in DPPC liposomes (Figure 3a). Neat DPPC liposomes showed $T_m$ of ∼42.3 °C in DPH studies, incorporation of 50% TamNHMe decreased $T_m$ to 34.6 °C, indicating TamNHMe molecules disorder packing of aliphatic alkyl chains, making them less rigid, leading to a lowering of $T_m$. LA-Tam$_1$-Am incorporation in DPPC membranes does not cause major changes in membrane rigidity in gel phase of membranes even up to 30% of doping


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(Figure 3b), whereas 50% incorporation increases membrane fluidity in gel phase to some extent. In the LC phase, more ordered arrangement was observed upon LA-$Tam_1$-Am doping as compared to neat DPPC liposomes. Temperature dependent fluorescence anisotropy studies showed that there is only an ∼2 °C change in phase transition temperature ($T_m$) even upon 50% doping of LA-$Tam_1$-Am in DPPC membranes. These observations indicate that LA-$Tam_1$-Am molecules gel well with DPPC membranes due to its lipidic nature of lithocholic acid as compared to the TamNHMe molecule; and free amine groups of LA-$Tam_1$-Am hydrogen bond with DPPC lipid molecules, therefore not allowing the phase transition temperature ($T_m$) to decrease (Figure 3b).

DCA-$Tam_2$-Am causes a systematic decrease in fluorescence anisotropy with an increase in the percentage of doping in DPPC membranes (Figure 3c) in the gel phase, indicating that DPH experiences disordered arrangement with an increase in doping due to liquification of membranes. Doping of DCA-$Tam_2$-Am in DPPC membranes fluidizes the gel phase as DCA-$Tam_2$-Am interferes with van der Waals interactions of hydrophobic tails of DPPC membranes. In the liquid crystalline phase, DCA-$Tam_2$-Am incorporation causes a little increase in anisotropy values of DPH, which further increases with an increase in doping, indicating a more ordered membrane structure in the liquid crystalline phase upon incorporation of DCA-$Tam_2$-Am. Temperature based studies (Figure 3c) on coliposomes of DCA-$Tam_2$-Am and DPPC showed no change in phase transition temperature ($T_m$) of coliposomes even on 50% doping of DCA-$Tam_2$-Am, indicating that doping of DCA-$Tam_2$-Am lowers the rigidity of membranes by disturbing the packing of aliphatic chains without decreasing the phase transition temperature of the coliposomes, which is evident from DSC studies as well as explained later.

Doping of CA-$Tam_3$-Am in DPPC membranes also decreases the fluorescence anisotropy as DPH experiences a more disordered environment in the gel phase of membranes. Doping (30% and 50%) of CA-$Tam_3$-Am causes a drastic decrease in membrane rigidity of DPPC membranes, and abolition of the phase transition temperature was observed at 30% incorporation (Figure 3d). In the liquid crystalline phase, there is no change in membrane fluidity upon incorporation of CA-$Tam_3$-Am, indicating that CA-$Tam_3$-Am does not cause any change in membrane packing of DPPC lipids in the liquid crystalline phase. CA-$Tam_3$-Am-DPPC coliposome studies indicate that CA-$Tam_3$-Am does not change the membrane organization in the fluid phase of DPPC membranes as DPH experiences a similar environment, whereas CA-$Tam_3$-Am fluidizes the gel phase of DPPC membranes upon its doping depending upon the percentage of incorporation. Comparing temperature based studies showed that CA-$Tam_3$-Am abolishes the phase transition temperature ($T_m$) of DPPC membranes and makes DPPC membranes highly fluid as compared to LA-$Tam_1$-Am and DCA-$Tam_2$-Am, where LA-$Tam_1$-Am gels very well in DPPC membranes and DCA-$Tam_2$-Am showed intermediate behavior.

The fluidity of model membranes in the gel phase strongly depends on the balance of the hydrophobicity of bile acids and the aromatic character of the TamNHMe molecule. The interactions between bile acid−tamoxifen conjugates and DPPC lipids depends upon the number and positioning of tamoxifen molecules conjugated to the bile acid. Conjugation of lithocholic acid to TamNHMe molecules provides concave hydrophobicity to the bile acids of these molecules. The hydrophobic concave side of the bile acid in LA-$Tam_1$-Am does not disturb the interactions between the staggered conformation of hydrophobic tails like TamNHMe, therefore causing less change in the phase transition and membrane fluidity as compared to TamNHMe. Among TamNHMe and bile acid−tamoxifen conjugates, DCA-$Tam_2$-Am and CA-$Tam_3$-Am fluidize membranes to the maximum extent as these molecules decrease the polarization in a concentration dependent way without affecting fluid phase of DPPC membranes. The differential effect of these molecules in the gel and fluid phase may be due to preferential accommodation of these molecules in the hydrophobic core of ordered membrane systems. The destabilizing/fluidizing effect on the model membrane is exerted due to conjugation of two or three TamNHMe molecules to bile acids, and not because of TamNHMe and bile acid alone, as TamNHMe and LA-$Tam_1$-Am do not fluidize the membranes to the same extent. DCA-$Tam_2$-Am having two tamoxifen molecules causes a more profound effect of membrane fluidity than TamNHMe and LA-$Tam_1$-Am, and less than CA-$Tam_3$-Am, which possesses three hydrophobic tamoxifen molecules and causes maximum destability in DPPC membranes. TamNHMe and bile acid−tamoxifen conjugates interact with DPPC liposomes and induce condensing and fluidizing effects that strongly depend on the temperature. Maximum differences in fluidizing effect were observed at the phase transition temperature ($T_m$).

DPH based fluorescence anisotropy studies showed that among three bile acid−tamoxifen conjugates, LA-$Tam_1$-Am causes minimal changes in membrane fluidity/rigidity of DPPC membranes in the gel and liquid crystalline phase and does not cause any change in $T_m$ values of DPPC membranes, whereas TamNHMe alone lowers the $T_m$ of DPPC membranes upon 50% doping. On the other hand, DCA-$Tam_2$-Am and CA-$Tam_3$-Am cause disordered arrangement of DPPC membranes upon doping by fluidizing the gel phase of DPPC membranes. Increased fluidity caused by DCA-$Tam_2$-Am and CA-$Tam_3$-Am is due to incorporation of more tamoxifen molecules conjugated with bile acid molecules, and these aromatic groups disturb interlipidic interactions between DPPC lipids, causing more fluidity, whereas LA-$Tam_1$-Am molecules seem to get gelled nicely with DPPC membranes, causing fewer disturbances in membrane rigidity. The order of membrane fluidity for bile acid−tamoxifen conjugates at 37 °C was found to be CA-$Tam_3$-Am > TamNHMe > DCA-$Tam_2$-Am > LA-$Tam_1$-Am. DCA-$Tam_2$-Am seems to cause the same amount of membrane fluidity as CA-$Tam_3$-Am upon 50% doping as two tamoxifen molecules are positioned in such a way that it can disturb DPPC membranes to same extent as CA-$Tam_3$-Am molecules. Overall DPH based anisotropy studies showed increase in membrane fluidity of DPPC membranes upon incorporation of bile acid−tamoxifen conjugates, which can make them ideal candidates for liposomal drug delivery. The membrane fluidity strongly depends upon the percentage of doping, and the number and positioning of tamoxifen molecules attached to bile acid molecules.

**Prodan Based Hydration Studies.** Interactions of drugs and drug delivery systems influences the water dynamics on the cell surface. Electrostatic interactions of drug molecules/delivery vehicles with lipid headgroups would displace water molecules from the membrane surface and increase the entropy of water molecules. Similarly, interactions of drug molecules or delivery vehicles with hydrophobic lipid membranes would fluidize the membrane and allow penetration of water


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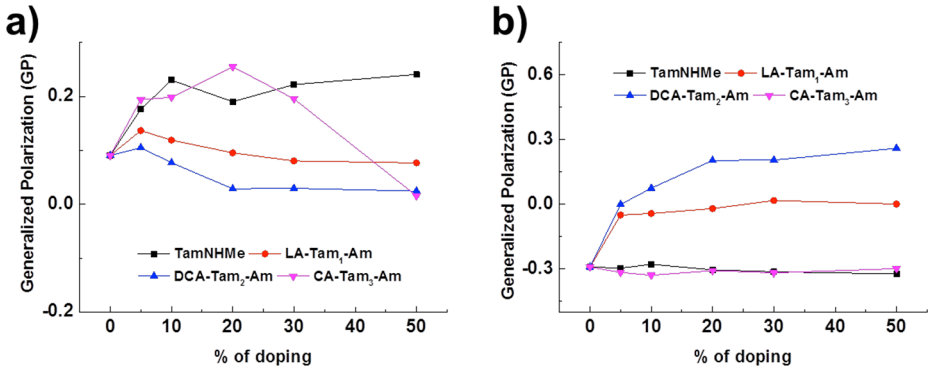


**Figure 4.** Change in generalized polarization (membrane hydration) of DPPC membranes in the gel phase (a) at 25 °C and liquid crystalline phase (b) at 55 °C upon incorporation of TamNHMe and bile acid−tamoxifen conjugates LA-Tam$_1$-Am, DCA-Tam$_2$-Am, and CA-Tam$_3$-Am.

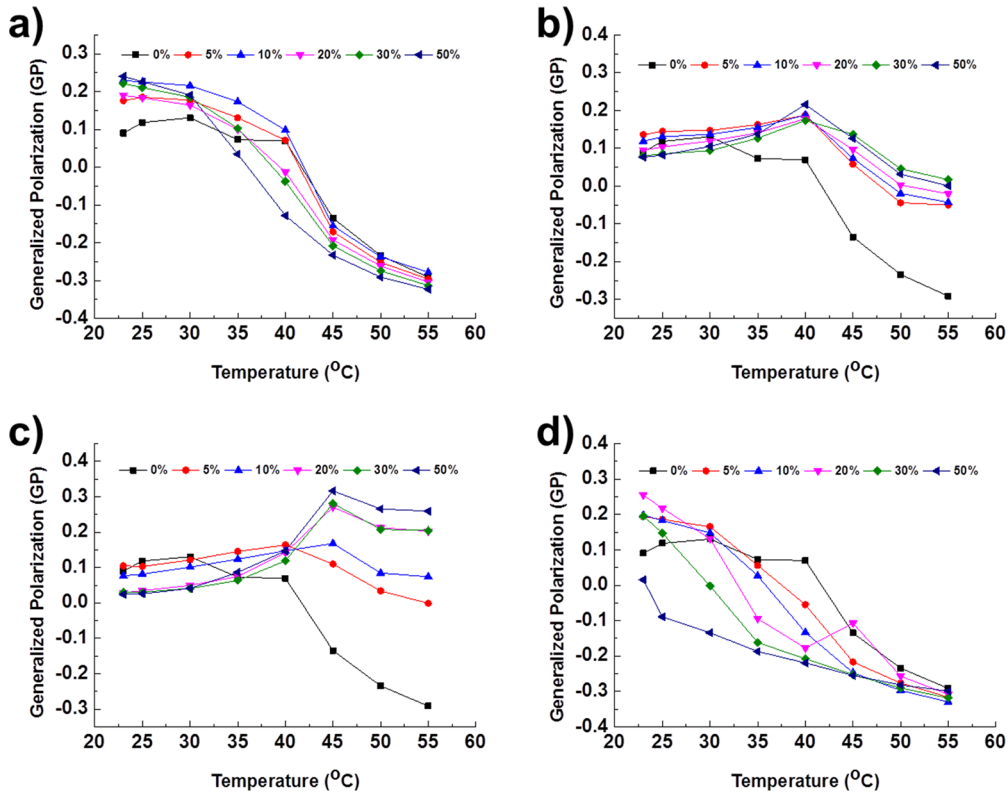


**Figure 5.** Effect of temperature on generalized polarization (membrane hydration) of DPPC membranes incorporated with different percentages of Tam-NHMe (a) and bile acid−tamoxifen conjugates LA-Tam$_1$-Am (b), DCA-Tam$_2$-Am (c), and CA-Tam$_3$-Am (d).

molecules in the lipid bilayer. The solvent relaxation behavior of water at the headgroup of membranes and of headgroups that penetrated into the bilayer can be studied by incorporating polarity sensitive fluorescent probes such as Prodan, Laurdan, Patman, etc.[31] Therefore, to understand dynamics of water molecules at the phospholipid surface upon incorporation of *N*-desmethylated tamoxifen (TamNHMe) and bile acid−tamoxifen conjugates, we performed Prodan based hydration studies.

In phospholipid liposomes, Prodan, like Laurdan and Patman, is tightly packed in the hydrophobic core by cooperative van der Waals interactions with lipid hydrocarbon chains having a fluorescent dimethylamino moiety residing at the level of the phospholipid headgroup. Prodan has the capability of getting located closer to the hydrated aqueous surface of liposomes and can sense more freely rotating water dipoles. The dipole moment of Prodan is due to a partial charge separation between dimethylamino and carbonyl residues and increases upon excitation. The reorientation of polar solvent molecules causes a decrease in the excited state of the probe, leading to a red shift in emission spectra in polar solvents whereas a blue shift is observed in apolar solvents. We have measured generalized polarization (GP) as a measure of the extent of hydration in coliposomes. Liposomes from DPPC doped with drugs in their gel states exhibit a high value of GP, which changes upon melting to the liquid-crystalline state due to changes in interactions among lipid molecules at different temperatures.

The arrangement of lipid molecules in phospholipid membranes influences the spectral shift of Prodan, the blue shift being in the gel phase whereas the red shift is in the liquid crystalline phase. A few water molecules are usually present at level of glycerol backbone in liposomes; the concentration and


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molecular dynamics of these water molecules change with an increase in temperature due to the increased concentration of water molecules near the glycerol backbone and their increased mobility. Doping of lipid−drug conjugates would influence the membrane surface hydration of DPPC lipids as hydrophobic interactions among lipid molecules and headgroup interactions would affect the water dynamics on membrane surface that would further depend on temperature.

Incorporation of bile acid−tamoxifen conjugates in DPPC membranes causes interesting changes in the lipid hydration of DPPC membranes. In the gel phase, doping of *N*-desmythylated tamoxifen (TamNHMe) decreases the membrane hydration of DPPC membranes as increased in generalized polarization (GP) is observed upon increase in doping of TamNHMe in membranes. The hydrophobic aromatic character of TamNHMe dominates and dehydrates the membrane surface, causing a decrease in membrane hydration. Doping of LA-$Tam_1$-Am does not cause any change in hydration of DPPC liposomes in the gel phase as these molecules seem to gel well with DPPC lipid molecules, as evident from DPH anisotropy studies as well. DCA-$Tam_2$-Am incorporation initially causes a minor increase in hydration of DPPC membranes in the gel phase (Figure 4). Doping of increasing amounts of DCA-$Tam_2$-Am decreases the generalized polarization and increases the hydration of coliposomes. An increase in hydration may be due to disorder of DPPC lipid molecules, as evident from DPH studies, allowing penetration of more water molecules into the bilayer and more hydration of the membrane surface. Doping of CA-$Tam_3$-Am as opposed to LA-$Tam_1$-Am and DCA-$Tam_2$-Am increases the generalized polarization or decreases hydration of DPPC vesicles up to 30% doping, whereas only 50% doping increases hydration of DPPC vesicles. More tamoxifen molecules attached to a cholic acid molecule cause a more hydrophobic environment and may not allow more hydration of DPPC membranes on its doping, whereas 50% doping exerts the maximum destability in DPPC membranes to make them more hydrated. In the liquid crystalline phase, TamNHMe and CA-$Tam_3$-Am do not cause any change in hydration of DPPC liposomes, whereas doping of LA-$Tam_1$-Am and DCA-$Tam_2$-Am decreases the hydration of coliposomes above the phase transition temperature ($T_m$). These studies indicate that LA-$Tam_1$-Am and DCA-$Tam_2$-Am dehydrate the fluid phase of DPPC membranes, whereas TamNHMe and CA-$Tam_3$-Am do not make any change in lipid hydration, making them gel completely with DPPC lipids in the fluid phase.

The influence of temperature on the membrane hydration of coliposomes was studied as shown in Figure 5. TamNHMe seems to make DPPC liposomes more hydrophobic in the gel state, making them less hydrated as compared to DPPC. An increase in TamNHMe doping causes a decrease in the phase transition temperature, which depends strongly upon the percentage of doping. Doping of 30% and 50% of TamNHMe decreases $T_m$ values of DPPC liposomes by only 2 °C. Temperature based studies showed unusual hydration behavior of coliposomes of DPPC and LA-$Tam_1$-Am (Figure 5b). With an increase in temperature to 40 °C, the phase transition temperature of liposomes, we have observed an increase in generalized polarization, indicating dehydration happening at the membrane interface, as opposed to the case of normal DPPC membranes where an increase in temperature usually causes more fluidity and a decrease in generalized polarization. This increase in generalized polarization with an increase in temperature in LA-$Tam_1$-Am incorporated liposomes indicates that LA-$Tam_1$-Am molecules gel well with DPPC membranes at the head region, making strong hydrogen bonding with phosphate headgroups of DPPC causing dehydration of membrane surface. A further increase in temperature above 40 °C causes a decrease in generalized polarization, indicating a more hydrated surface of membranes in the LC phase. In the LC phase, an increase in incorporation of LA-$Tam_1$-Am causes an increase in generalized polarization that indicates that coliposomes with high amounts of LA-$Tam_1$-Am cause a less hydrated membrane surface as compared to DPPC membranes. Putting all this together, coliposomes of LA-$Tam_1$-Am with DPPC are less hydrated in the LC phase as compared to DPPC membranes, and at the phase transition temperature coliposomes bear a maximum dehydrated surface.

DPPC membranes experience highly hydrated surfaces after comixing with DCA-$Tam_2$-Am, and this hydration increases with an increase in percentage of incorporation. In the gel phase of DPPC-DCA-$Tam_2$-Am coliposomes, a decrease in generalized polarization or an increase in membrane hydration is observed as these liposomes bear more fluidity as compared to neat liposomes, as shown by DPH studies as well. With an increase in temperature (Figure 5c), we have observed an increase in generalized polarization, a trend similarly observed for LA-$Tam_1$-Am incorporated liposomes, causing a dehydrated lipid surface. The increase in generalized polarization or decrease in membrane hydration with temperature strongly depends upon the percent incorporation of DCA-$Tam_2$-Am in DPPC membranes. With an increase in percent incorporation, we have observed a steep increase in generalized polarization or decrease in membrane hydration, indicating that high amounts of incorporation of DCA-$Tam_2$-Am cause more dehydrated membrane surfaces with an increase in temperature. In the LC phase, fewer membrane hydrated surfaces were observed with an increase in DCA-$Tam_2$-Am loading in DPPC membranes. Coliposomes of DPPC with higher doping percentages of DCA-$Tam_2$-Am make the liposomes dry.

With an increase in incorporation of CA-$Tam_3$-Am, there is an increase in generalized polarization of Prodan up to 20%, whereas a further increase in doping decreases the generalized polarization. This initial increase followed by a decrease in membrane hydration upon incorporation of CA-$Tam_3$-Am indicates that upon initial doping, strong hydrogen bonding between amine functionality of CA-$Tam_3$-Am and phospholipids dehydrate the lipid surface, as observed during DPH studies as well, whereas a further increase in incorporation fluidizes the membrane in the gel phase due to more doping of the aromatic character of CA-$Tam_3$-Am. Temperature based studies (Figure 5d) showed a lowering of the phase transition temperature of the coliposomes upon incorporation of CA-$Tam_3$-Am, ultimately abolishing the $T_m$ upon 50% doping, and a similar trend was observed from DPH based anisotropy studies as well.

Hydration at the surface on liposomes having DPPC lipids and bile acid−tamoxifen conjugates strongly depends on the nature of the bile acid−tamoxifen conjugate. LA-$Tam_1$-Am and DCA-$Tam_2$-Am pack with DPPC lipids using the concave hydrophobic surface of the bile acid packed with DPPC lipids and hydrogen bond with the phospholipid using the amino headgroup, making the lipid dry. With an increase in temperature these interactions become stronger and dehydrate the liposome surface. On the other hand, incorporation of CA-$Tam_3$-Am destabilizes or fluidizes the DPPC lipid membranes


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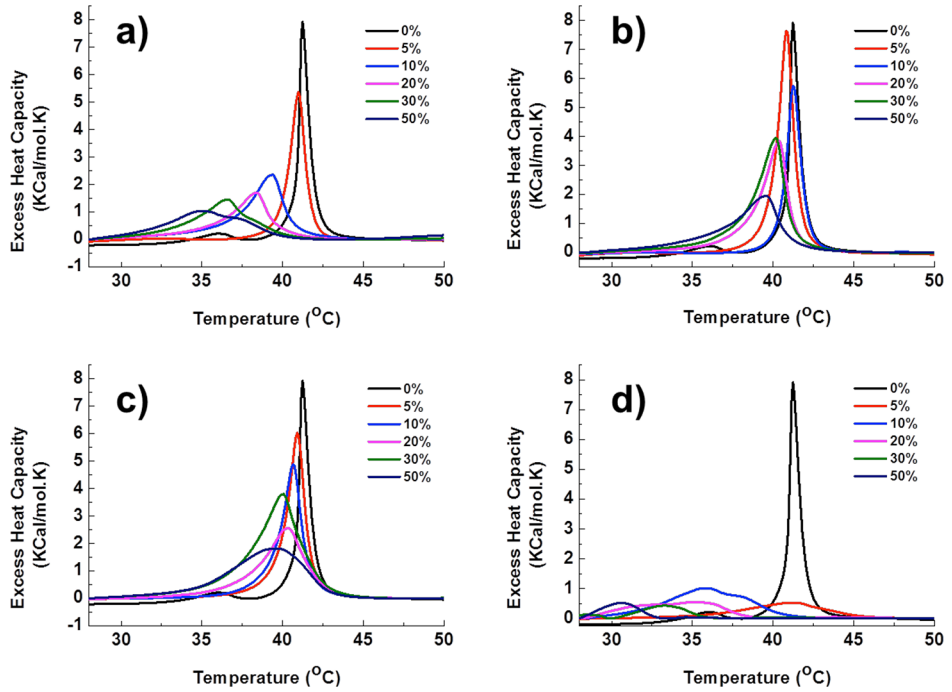


**Figure 6.** Differential scanning calorimetric transition graphs of neat DPPC liposomes and upon doping of different percentages of TamNHMe (a) and bile acid–tamoxifen conjugates LA-Tam$_1$-Am (b), DCA-Tam$_2$-Am (c), and CA-Tam$_3$-Am (d).

due to the higher aromatic character of the lipid molecule. This destabilization of liposomes brings more penetration of water molecules at the headgroup surface, making the liposomes wet. Therefore, the balance of hydrophobicity of the bile acid and aromaticity of tamoxifen molecules plays a critical role in the hydration of membranes. Overall, Prodan based hydration studies showed that LA-Tam$_1$-Am and DCA-Tam$_2$-Am dehydrate the membrane surface depending on the temperature, the maximum dehydration at the phase transition temperature. CA-Tam$_3$-Am on other hand makes DPPC liposomes highly hydrated in the gel phase.

**Differential Scanning Calorimetry.** Differential scanning calorimetry presents a nonperturbative technique to understand the aggregation behavior of natural and synthetic lipids, effects of different biomolecules/drugs with biological membranes, and effects of different synthetic lipids/polymers with model cell membranes, etc.[32] Model membranes such as liposomes undergo a phase transition from the gel-like phase to the LC phase at a particular temperature called the phase transition temperature ($T_m$) that strongly depends on the nature of the lipids. Interactions of biomolecules, lipids, and drugs with these model membranes affect the phase behavior of the liposomes and changes their thermodynamic properties, which play an important role in the drug diffusion/drug delivery properties of molecules. Therefore, we studied interactions of bioactive bile acid–tamoxifen conjugates with model DPPC membranes using differential scanning calorimetry, as shown in Figures 6 and 7 and Table 1.

We studied interaction of *N*-desmethylated tamoxifen (TamNHMe), LA-Tam$_1$-Am, DCA-Tam$_2$-Am, and CA-Tam$_3$-Am with DPPC membranes upon doping of these molecules at different percentages, as shown in Figures 6 and 7. We observed a prominent decrease in the phase transition temperature of DPPC with an increase in percentage doping of TamNHMe and bile acid–tamoxifen conjugates that strongly depends on the bile acid, the number of tamoxifen

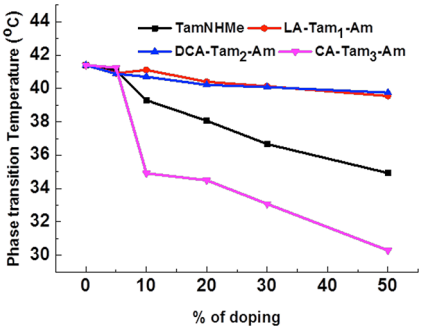


**Figure 7.** Effect of incorporation of different percentages of TamNHMe and bile acid–tamoxifen conjugates LA-Tam$_1$-Am, DCA-Tam$_2$-Am, and CA-Tam$_3$-Am on the phase transition temperature ($T_m$) of DPPC liposomes.

molecules attached, and the percentage of doping. Doping of increased percentages of these conjugates caused progressive broadening of the gel-to-liquid phase transition of DPPC. The similar kind of phase transition behavior was observed in fluorescence studies of DPPC coliposomes with different percentages of bile acid–tamoxifen conjugates using 1% DPH as the fluorescent probe.

Incorporation of TamNHMe in DPPC liposomes decreased the phase transition temperature ($T_m$) in a linear fashion with an increase in the percentage of doping. TamNHMe with its polar aminomethyl functionality of the side chain may strongly orient in the phosphocholine headgroup, whereas the rest of the aromatic backbone dynamically interacts with acyl chains of DPPC, causing a decrease in the main phase transition temperature ($T_m$) with an increase in the percentage of doping (Figure 6a). Increasing the percentage incorporation of LA-Tam$_1$-Am and DCA-Tam$_2$-Am in DPPC membranes caused a minor decrease in the phase transition temperature ($T_m$) of DPPC. Therefore, comparisons of TamNHMe with LA-Tam$_1$-


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**Table 1. Thermodynamic Characterization of Phase Transition Exhibited by DPPC and Its Co-aggregates with TamNHMe and Bile Acid−tamoxifen Conjugates As Determined from Differential Scanning Calorimetry**

| compound | % in DPPC[a] | $T_m$[b] (°C) | $\Delta H_c$ (kcal/mol) | $\Delta S$ [cal/(mol K)] | $\Delta H_{vH}$ (kcal/mol) | CU[c] | fwhm[d] | $C_p^{max}$[e] |
|---|---|---|---|---|---|---|---|---|
| DPPC | | 41.40 | 7.92 | 25.20 | | | | |
| TamNHMe | 5.00 | 41.08 | 7.48 | 23.80 | 482.60 | 64.48 | 1.31 | 4.85 |
| | 10.00 | 39.30 | 8.24 | 26.40 | 171.07 | 20.75 | 2.97 | 2.02 |
| | 20.00 | 38.06 | 6.39 | 20.50 | 140.41 | 21.99 | 3.25 | 1.41 |
| | 30.00 | 36.67 | 6.70 | 21.60 | 104.99 | 15.68 | 4.40 | 1.22 |
| | 50.00 | 34.93 | 6.94 | 22.50 | 58.49 | 8.42 | 5.01 | 0.90 |
| LA-Tam$_1$-Am | 5.00 | 40.92 | 11.29 | 35.90 | 449.26 | 39.80 | 1.38 | 6.82 |
| | 10.00 | 41.11 | 7.26 | 24.00 | 509.01 | 70.11 | 1.21 | 5.24 |
| | 20.00 | 40.40 | 9.62 | 30.70 | 223.83 | 23.28 | 2.22 | 3.33 |
| | 30.00 | 40.13 | 10.83 | 34.60 | 184.56 | 17.04 | 2.49 | 3.38 |
| | 50.00 | 39.55 | 8.42 | 26.90 | 90.11 | 10.70 | 3.95 | 1.63 |
| DCA-Tam$_2$-Am | 5.00 | 40.88 | 10.65 | 33.90 | 366.98 | 34.45 | 1.54 | 5.26 |
| | 10.00 | 40.70 | 9.95 | 31.70 | 296.07 | 29.74 | 1.83 | 4.19 |
| | 20.00 | 40.22 | 9.55 | 30.50 | 152.65 | 15.98 | 3.47 | 2.26 |
| | 30.00 | 40.08 | 15.01 | 47.90 | 128.41 | 8.55 | 3.66 | 3.26 |
| | 50.00 | 39.75 | 10.58 | 33.80 | 78.38 | 7.41 | 5.18 | 1.78 |
| CA-Tam$_3$-Am | 5.00 | 41.25 | 3.37 | 10.70 | 107.68 | 31.95 | 6.08 | 0.49 |
| | 10.00 | 34.91 | 6.45 | 20.90 | 100.05 | 15.52 | 5.87 | 0.95 |
| | 20.00 | 34.50 | 3.70 | 12.00 | 94.87 | 25.61 | 6.63 | 0.56 |
| | 30.00 | 33.08 | 1.46 | 4.80 | 164.59 | 112.92 | 3.06 | 0.42 |
| | 50.00 | 30.30 | 1.45 | 4.80 | 158.27 | 109.46 | 2.59 | 0.52 |

[a]See text for experimental details on DSC; (DPPC) = 0.5 mg/mL. [b]Accuracy of $T_m$ was ±1.0 °C. [c]Size of cooperativity unit. [d]Full width at half-maximum. [e]Maximum point of specific heat capacity.

Am doping studies indicate that TamNHMe causes more disruptions in packing of DPPC lipids, whereas conjugation of TamNHMe with lithocholic acid in LA-Tam$_1$-Am allows the molecule to pack efficiently in DPPC lipids. Similarly, doping of DCA-Tam$_2$-Am could make only a minor change in the phase transition temperature ($T_m$) of DPPC membranes even with 50% doping, although we have observed much-broadened peaks as compared to doping of LA-Tam$_1$-Am. These studies indicate that free TamNHMe is not able to gel with hydrophobic interactions of alkyl chains of phospholipids and its aromatic character disrupts these interactions to a greater extent, making the phase transition temperature ∼37 °C and ∼35 °C upon 30% and 50% doping, respectively. On the other hand, upon doping of LA-Tam$_1$-Am and DCA-Tam$_2$-Am having one and two molecules of TamNHMe conjugated to bile acids, there is only a decrease of ∼1 °C in the phase transition temperature. Similar observations were made from DPH based anisotropy studies, indicating that LA-Tam$_1$-Am and DCA-Tam$_2$-Am make the gel phase of DPPC lipids more fluid but do not change the phase transition temperature of the liposomes. Incorporation of CA-Tam$_3$-Am in DPPC liposomes leads to a steep decrease in phase transition temperature ($T_m$) (starting from 10% doping) as well as broadening of peak that strongly depends on percentage of doping. Doping of 10% of CA-Tam$_3$-Am decreases the phase transition temperature to 35 °C and 50% doping makes it 30 °C, which can be abolished upon further doping. These studies indicate that CA-Tam$_3$-Am molecules cause maximum disorderliness among all molecules. One possibility is CA-Tam$_3$-Am, with three planar tamoxifen molecules that cooperatively self-assemble in DPPC membranes, causing more disorderliness in the gel phase packing of acyl chains of DPPC (Figure 7). Concentrations of these bile acid−tamoxifen conjugates required for complete abolition of the main phase transition varied significantly as compared to cholesterol and other bile acid−tamoxifen conjugates as even with incorporation of 50% of TamNHMe, LA-Tam$_1$-Am, and DCA-Tam$_2$-Am conjugates, we have not observed abolition of the main phase transition peak of DPPC in liposomes.

Staggered conformation of hydrophobic tails of DPPC lipids makes strong hydrophobic van der Waals interactions among themselves, which makes its liposomes have a phase transition temperature ($T_m$). Incorporation of TamNHMe diminishes the interactions between hydrophobic tails of DPPC, lowering the phase transition temperature ($T_m$) of mixed liposomes, whereas hydrophobic interactions between aromatic moieties of TamNHMe and DPPC lipids does not allow the fluidizing of membranes as observed in DPH anisotropy studies. Conjugation of lithocholic acid and deoxycholic acid to tamoxifen molecules provides concave hydrophobicity of bile acids to these molecules. Hydrophobic concave side of bile acid in LA-Tam$_1$-Am and DCA-Tam$_2$-Am does not disturb the interactions between staggered conformations of hydrophobic tails like TamNHMe, therefore causing less change in phase transition as compared to TamNHMe but an increase in aromaticity on tamoxifen conjugates LA-Tam$_1$-Am and DCA-Tam$_2$-Am dehydrate the membranes. In the case of CA-Tam$_3$-Am, conjugation of three tamoxifen molecules increases the aromatic character of the molecule, and causes maximum destabilization of hydrophobic interactions of DPPC lipids leading to diminishing the phase transition and fluidity of the membranes. Therefore, the critical balance of bile acid hydrophobicity and aromatic character of TamNHMe controls the phase transition and membrane fluidity of the membranes.

Comparison of calorimetric enthalpies associated with melting of hydrophobic chains in DPPC lipids and its coliposomes with bile acid−tamoxifen conjugates showed that there is a decrease in enthalpies and entropies of transition upon an increase in the percentage of doping in the case of TamNHMe and CA-Tam$_3$-Am due to progressive broadening of the gel to liquid phase transition of liposomes. On the other


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hand doping of LA-Tam$_1$-Am and DCA-Tam$_2$-Am shows a complex trend in enthalpies and entropies of transition. The complex trend observed in the case of LA-Tam$_1$-Am and DCA-Tam$_2$-Am is evident from the fact that doping of these molecules does not decrease the phase transition temperature of DPPC liposomes, and their mixing with DPPC lipids is less effective in quenching the order of DPPC lipid chains. The presence of impurities or incorporation of drug molecules in lipid bilayers results in broadening of phase transition peaks usually expressed by the van't Hoff equation. We calculated van't Hoff enthalpies from $C_p^{max}$, calorimetric enthalpies, and mole fractions of doped drugs. Increase in doping percentage of TamNHMe and bile acid- tamoxifen conjugates lead to progressive decrease in van't Hoff enthalpies. However, van't Hoff enthalpies in the case of lipid bilayers were much larger than the calorimetric enthalpies because of intermolecular cooperation. The size of such intermolecular cooperation or cooperativity unit (CU or $n$) is a cluster of self-assembled molecules more clearly phospholipids in this case, in the gel to liquid ordered phase transition. The size of the transition cooperativity unit mainly depends on the full width at half-maximum (fwhm or $\Delta T_{1/2}$), sharpness of the main phase transition peak, the phase transition temperature ($T_m$), and the height of the main phase transition peak, i.e., the point of maximum excess heat capacity ($C_p^{max}$). There is an inverse relation between CU and fwhm ($\Delta T_{1/2}$), or a direct relation between CU and the sharpness of main phase transition peak.[33] As shown in Table 1, doping of TamNHMe and bile acid−tamoxifen conjugates in general showed a decrease in the cooperation of DPPC lipids with an increase in the percentage of doping because of the general increase in the full width at half-maximum and the decrease in the height of the transition peak. Less cooperativity observed in the case of an increase in percentage doping of bile acid−tamoxifen conjugates may be due to more aromatic TamNHMe conjugated to bile acids that disrupts the lipid interactions in these coliposomes, making the transition less cooperative.

Phase transitions of coliposomes of DPPC lipids and TamNHMe or bile acid−tamoxifen conjugates are the sum of two component peaks. The complex nature of these transitions possessing a sharp peak and broad peak becomes more evident upon doping of 30% and 50% of TamNHMe, and also in the case of 10% doping of CA-Tam$_3$-Am. A 30% doping of TamNHMe causes a phase transition temperature at 37 °C along with a broader transition at same region, and similarly 50% doping of TamNHMe makes a transition at 35 °C along with a broader transition. In the case of doping of LA-Tam$_1$-Am and DCA-Tam$_2$-Am, the presence of two transitions is less evident except in the case of 50% doping of LA-Tam$_1$-Am and DCA-Tam$_2$-Am. The 10% doping of CA-Tam$_3$-Am showed clearly a mixture of two transitions at 37 and 39 °C. The observation of two transitions indicates the presence of different domains upon the incorporation of TamNHMe and bile acid−tamoxifen conjugates in DPPC liposomes. Doping of TamNHMe and bile acid−tamoxifen conjugates may induce the formation of two different kinds of domains: (1) DPPC enriched domains having TamNHMe or bile acid−tamoxifen conjugate or (2) TamNHMe or bile acid−tamoxifen enriched domains. DPPC enriched domains are responsible for sharp transitions of coliposomes whereas broadened transitions could arise of other bile acid enriched domains. The presence of two domains was clearly evident from the transition of 10% doping of CA-Tam$_3$-Am, indicating that these coliposomes have a domain enriched in CA-Tam$_3$-Am molecules where aromatic tamoxifen molecules can get stacked with each other. The possibility of this tamoxifen stacking decreases from CA-Tam$_3$-Am to DCA-Tam$_2$-Am to LA-Tam$_1$-Am.

Overall, calorimetric studies showed that introduction of TamNHMe in DPPC liposomes causes membrane perturbation and lowering of phase transition temperature depending on the percentage of doping. Lowering the phase transition was not observed during LA-Tam$_1$-Am and DCA-Tam$_2$-Am doping, indicating that alone TamNHMe causes more distortions whereas bile acid conjugation makes the membranes less disturbed in DPPC membranes. Cholic acid based tamoxifen conjugate CA-Tam$_3$-Am was found to be highly effective in lowering the phase transition temperature and caused maximum disorder.

## CONCLUSIONS

Interactions of drugs and lipid−drug conjugates with DPPC membranes are critical in understanding the mechanism of action for different drug molecules and understanding their interactions with the cell surface. Therefore, we studied interactions of anticancer bile acid−tamoxifen conjugates, which are potent lipid−drug conjugates for breast cancer therapy, with model DPPC membranes. Fluorescence anisotropy studies showed the dependence of membrane fluidity on the percentage of doping of bile acid−tamoxifen conjugates in DPPC membranes and the number and position of tamoxifen molecules attached to the bile acid molecules, as DCA-Tam$_2$-Am and CA-Tam$_3$-Am were found to distort membranes, causing high fluidity in the gel phase. Prodan based hydration studies showed that incorporation of LA-Tam$_1$-Am and DCA-Tam$_2$-Am with DPPC makes the membranes dry at the phase transition temperature ($T_m$) and in the LC phase, whereas CA-Tam$_3$-Am makes the liposomes highly hydrated in the gel phase. DSC studies showed that the thermotropic behavior of coliposomes depends strongly on the bile acid−tamoxifen conjugate and the percentage of incorporation of compounds. CA-Tam$_3$-Am even at 10% doping lowered the phase transition temperature, whereas LA-Tam$_1$-Am and DCA-Tam$_2$-Am do not make any change in $T_m$ as opposed to TamNHMe alone that lowers $T_m$ upon its doping. TamNHMe decreases the phase transition temperature without any subtle changes in the fluidity of the membrane. Both LA-Tam$_1$-Am and DCA-Tam$_2$-Am have not affected the phase transition temperature, but they influenced the order of fluidity of the membrane in an incremental manner. On the other hand, CA-Tam$_3$-Am lowered the phase transition temperature and increased the membrane fluidity. CA-Tam$_3$-Am induces maximum distortions in cooperative acyl chain packing of DPPC lipids, causing maximum lowering in $T_m$ and maximum hydration, whereas LA-Tam$_1$-Am and DCA-Tam$_2$-Am conjugates gel well with the membranes and dehydrates the membranes without a change in the phase transition temperature ($T_m$). These changes in dynamic properties of lipids indicate their differential interactions with membrane lipids, depending on the structural features of bile acid−drug conjugates, which is important in the operation of multiple cellular effects and the mechanism of actions of tamoxifen and bile acid−tamoxifen conjugates for anticancer therapy.


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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

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## REFERENCES


(1) Vereb, G.; Szollosi, J.; Matko, J.; Nagy, P.; Farkas, T.; Vigh, L.; Matyus, L.; Waldmann, T. A.; Damjanovich, S. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 8053−8058.

(2) Mouritsen, O. G.; Jorgensen, K. *Pharm. Res.* **1998**, *15*, 1507−1519.

(3) Seydel, J. K.; Coats, E. A.; Cordes, H. P.; Wiese, M. *Arch. Pharm (Weinheim)* **1994**, *327*, 601−610.

(4) Seydel, J. K., Wiese, M., Eds. *Drug-membrane Interactions*; Wiley-VCH: Weinheim, 2002.

(5) Van Meer, G.; Voelker, D. R.; Feigenson, G. W. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 112−124.

(6) Tamm, L. K.; McConnell, H. M. *Biophys. J.* **1985**, *47*, 105−113.

(7) Landsberg, M. J.; Ruggles, J. L.; Hussein, W. M.; McGeary, R. P.; Gentle, I. R.; Hankamer, B. *Langmuir* **2010**, *26*, 18868−18873.

(8) Eytan, G. D. *Biochim. Biophys. Acta* **1982**, *694*, 185−202.

(9) Peetla, C.; Stine, A.; Labhasetwar, V. *Mol. Pharmaceutics* **2009**, *6*, 1264−1276.

(10) Grancelli, A.; Morros, A.; Cabanas, M. E.; Domenech, O.; Merino, S.; Vazquez, J. L.; Montero, M. T.; Vinas, M.; Hernandez-Borrell, J. *Langmuir* **2002**, *18*, 9177−9182.

(11) Barcelo, F.; Prades, J.; Funari, S. S.; Frau, J.; Alemany, R.; Escriba, P. V. *Mol. Membr. Biol.* **2004**, *21*, 261−268.

(12) Corvis, Y.; Barzyk, W.; Brezesinski, G.; Mrabet, N.; Badis, M.; Hecht, S.; Rogalska, E. *Langmuir* **2006**, *22*, 7701−7711.

(13) Agasosler, A. V.; Tungodden, L. M.; Cejka, D.; Bakstad, E.; Sydnes, L. K.; Holmsen, H.. *Biochem. Pharmacol.* **2001**, *61*, 817−825.

(14) Feng, S. S.; Gong, K.; Chew, J. *Langmuir* **2002**, *18*, 4061−4070.

(15) Lucio, M.; Bringezu, F.; Reis, S.; Lima, J. F. C.; Brezesinski, G. *Langmuir* **2008**, *24*, 4132−4139.

(16) Park, Y.; Franses, E. I. *Langmuir* **2010**, *26*, 6932−6942.

(17) Gzyl-Malcher, B.; Filek, M.; Brezesinski, G. *Langmuir* **2011**, *27*, 10886−10893.

(18) Jordan, V. C. *Nat. Rev. Drug. Discov.* **2003**, *2*, 205−213.

(19) Clarke, R.; Van den Berg, H. W.; Murphy, R. F. *J. Natl. Cancer Inst.* **1990**, *82*, 1702−1705.

(20) Custddio, J. B. A.; Almeida, L. M.; Madeira, V. M. C. *Biochim. Biophys. Acta* **1993**, *1150*, 123−129.

(21) Mukhopadhyay, S.; Maitra, U. *Curr. Sci.* **2004**, *87*, 1666−1683.

(22) Chu, B.-S.; Gunning, A. P.; Rich, G. T.; Ridout, M. J.; Faulks, R. M.; Wickham, M. S. J.; Morris, V. J.; Wilde, P. J. *Langmuir* **2010**, *26*, 9782−9793.

(23) Balakrishnan, A.; Polli, J. E. *Mol. Pharmaceutics* **2006**, *3*, 223−230.

(24) Mohapatra, M.; Mishra, A. K. *Langmuir* **2011**, *27*, 13461−13467.

(25) Bernsdorff, C.; Winter, R. *J. Phys. Chem. B* **2003**, *107*, 10658−10664.

(26) Bhattacharya, S.; Bajaj, A. *Biochim. Biophys. Acta* **2008**, *1778*, 2225−2223.

(27) Bhattacharya, S.; Bajaj, A. *J. Phys. Chem. B* **2007**, *111*, 13511−13519.

(28) Bhattacharya, S.; Bajaj, A. *J. Phys. Chem. B* **2007**, *111*, 2463−2472.

(29) Ghosh, Y. K.; Indi, S. S.; Bhattacharya, S. *J. Phys. Chem. B* **2001**, *105*, 10257−10265.

(30) Sturtevant, J. M. *Proc. Natl. Acad. Sci. U. S. A.* **1982**, *79*, 3963−3967.

(31) Parasassi, T.; Krasnowska, E. K.; Bagatolli, L.; Gratton, E. *J. Fluoresc.* **1998**, *8*, 365−373.

(32) Hinz, H. J.; Sturtevant, J. M. *J. Biol. Chem.* **1972**, *247*, 6071−6075.

(33) Cong, W.; Liu, Q.; Liang, Q.; Wang, Y.; Luo, G. *Biophys. Chem.* **2009**, *143*, 154−160.