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Unraveling the Impact of Hydroxylation on Interactions of Bile Acid Cationic Lipids with Model Membranes by In-depth Calorimetry Studies

Manish Singh and Avinash Bajaj*

The Laboratory of Nanotechnology and Chemical Biology, Regional Centre for Biotechnology, 180 Udyog Vihar, Phase I, Gurgaon-122016, Haryana, India. E-mail: bajaj@rcb.res.in

Abstract: We used eight bile acid cationic lipids differing in number of hydroxyl groups and performed in-depth differential scanning calorimetry studies on model membranes doped with different percentage of these cationic bile acids. These studies revealed that number and positioning of free hydroxyl groups on bile acids modulate the phase transition and co-operativity of membranes. Lithocholic acid based cationic lipids having no free hydroxyl group, gel well with dipalmitoyl phosphatidylcholine (DPPC) membranes. Chenodeoxycholic acid lipids having one free hydroxyl group at 7'-carbon position disrupts the membranes and lowers its co-operativity. Deoxycholic acid and cholic acid based cationic lipids have free hydroxyl group at 12'-carbon position, and at 7'- and 12'-carbon positions respectively. Doping of these lipids at high concentrations increases co-operativity of membranes suggesting that these lipids might induce self-assembly in DPPC membranes. These differential modes of interactions between cationic lipids and model membranes would help in future for exploring their use in DNA/drug delivery.

Introduction: Cellular functions of mammalian cells depend on structure, composition, and dynamics of membranes.[1] Cellular membranes also help in protection, control of transport of ions, nutrients, and serve as target of many drugs.[1] Phospholipids, sphingolipids, and sterols build up the matrix of cellular membranes. Composition of cellular membranes and its interactions with external stimuli is critical for cell growth, differentiation, development, and cellular movement.

Interactions of membranes with drugs, drug delivery systems, and antibacterial peptides determine the efficacies of these molecules.[2] Many physico-chemical studies of interactions of model membranes with different biomolecules have been performed using differential scanning calorimetry,[3] anisotropy studies,[4] and hydration dynamics.[5] Toimil *et al* studies the influence of subphase temperatue on mixed monolayers.[6] Lucio and co-workers studied the influence of anti-inflammatory drugs on membrane fluidity by fluorescence anisotopy measurements.[7] Recently influence of dicationic gemini surfactants on structure and order of DODAB bilayers was studied by Almeida and co-workers.[8] Strzalka and co-workers studied in-depth calorimetric studies to understand the influence of different structures of carotenoids on dynamics of membranes.[9]

Cationic amphiphiles have been explored as delivery agents of genetic material to eukaryotic cells,[10] drug encapsulation and delivery,[11] and as antitumor,[12] antibacterial,[13] and antiviral agents.[14] The bioactivity of these cationic lipids is strongly contingent on nature of the head group and hydrophobicity of lipids. Bhattacharya *et al* studied the interactions of cationic lipids[15] and cationic lipidated polymer[16] with model membranes to uncover the insights for efficient DNA delivery. Molecular dynamic simulations have also been performed to understand the cationic lipid-phospholipid interactions.[17] Therefore studies of interactions of cationic lipids with membranes are important to unravel the activity and mechanistic insights of these amphiphiles.

Bile acids are facial amphiphiles that possess different number of hydroxyl groups on concave side of their steroidal backbone.[18] The interactions of the bile acids with colon epithelium is critical for fat absorption, bile acid re-circulation, and pathogenesis of diseases like inflammation, ulcer, and

colon cancer. Mishra and co-workers have studied the effect of sub-micellar concentrations of conjugated and unconjugated bile salts on lipid bilayer membranes.[19] They have shown that sub-micellar bile salt concentration induces hydration of lipid bilayer membrane in the core region using steady state and time-resolved fluorescence spectroscopy. Bile acid based cationic lipids have also been explored for DNA delivery applications.[20] Roessler *et al* has engineered oligoarginine-conjugated bile acids and studied their transfection efficacies.[21] Therefore, interactions of bile acid cationic lipids with model membranes would help us in unraveling the differential activity of these amphiphiles on interactions with biological systems. It would also help in understanding the influence of subtle changes, like number of free hydroxyl groups in structures of bile acids, on interactions with biological membranes.

In this paper, we used eight cationic lipids (Figure 1) based on lithocholic acid (LCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and cholic acid (CA). We conjugated cationic head groups trimethyl amine (LCA-TMA, CDCA-TMA, DCA-TMA, CA-TMA) and dimethylamino ethanol (LCA-DME, CDCA-DME, DCA-DME, CA-DME) to 3'-OH group of bile acids (Figure 1). LCA based cationic lipids LCA-TMA and LCA-DME donot possess any free hydroxyl group. CDCA and DCA based cationic lipids have one free hydroxyl group at 7'- (CDCA-TMA, CDCA-DME) and 12'-carbon position (DCA-TMA, DCA-DME) respectively. CA based cationic lipids (CA-TMA, CA-DME) hold two free hydroxyl groups at 7'-and 12'-carbon positions. We studied the interactions of these bile acid based cationic lipids with model membranes using differential scanning calorimetry studies. These studies revealed that number of free hydroxyl groups and their positioning dramatically influences the interactions of these bile acid based cationic lipids with model membranes irrespective of the cationic head group.

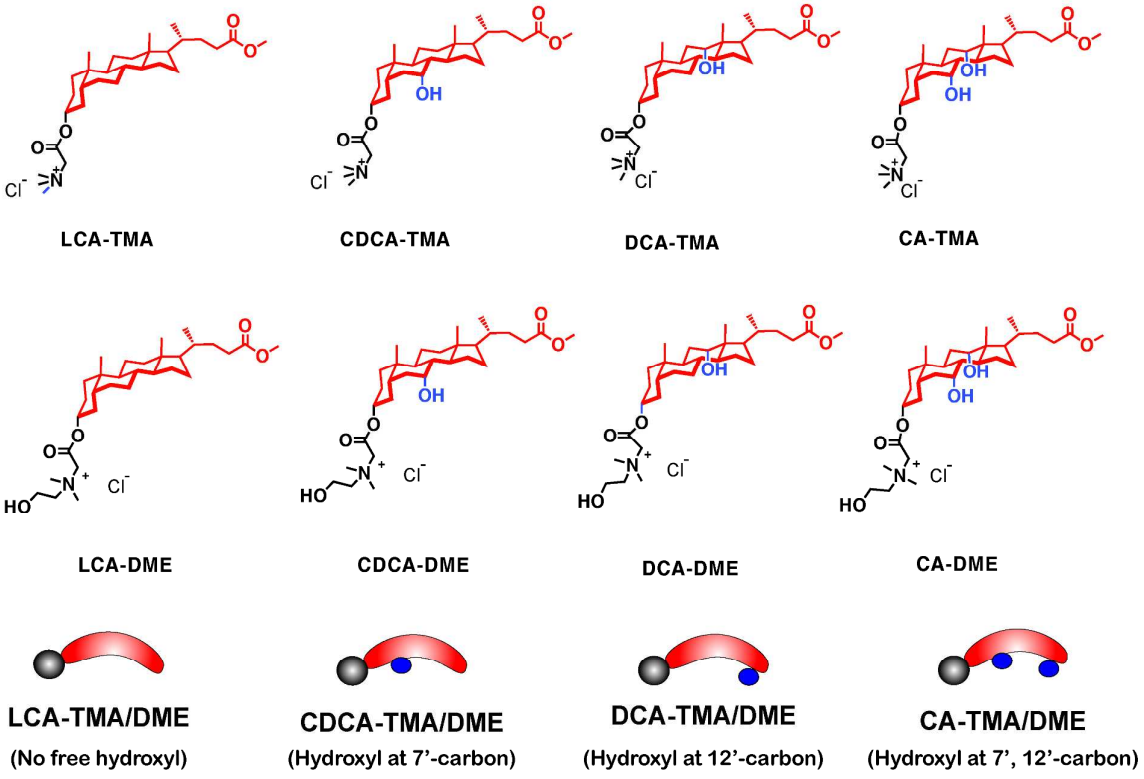

Figure 1. Molecular structures and schematic presentation of bile acid based cationic lipids studied.

Methods

General: All cationic bile acids derivatives used in these experiment were synthesized and characterized by NMR and mass spectroscopy. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and was used as received. Double filtered Milli-Q water was used for samples preparation.

Liposome preparation[22]: Liposomes were prepared by traditional thin-film method. Briefly, DPPC and appropriate amount cationic bile acids (5, 10, 20, 30 and 50 mol% of DPPC) were dissolved in chloroform/methanol. Organic solvent was removed with stream of dry nitrogen under rotary motion and finally solvent were evaporated under vacum to obtain lipid thin film. Each lipid film sample was kept for hydration at 4°C for 12h. The suspension was heated to 70 °C for 10 min, frozen to ice for 10 min and then vortex for 5 min. Each sample was subjected to repeat four-five freeze thaw cycles to ensure optimal hydration and sonicated to get unilamellar vesicles.[23]

Differential scanning calorimetry:[22] The calorimetry studies were done on Nano Scientific high-sensitivity differential scanning calorimeter from TA Instruments. For all DSC samples, scan rate of 1 °C/min was used to ensures a minimum endotherm curve distortion due to instrumental time lag. Aliquots of 750 μL were withdrawn for DSC analyses for studying the main phase transition. All samples were degassed under 230 mm Hg pressure prior to being run for 30 min. Data acquired were analyzed with NanoAnalyzer (TA Instruments), and plotted with Origin version 8.5 Pro (Origin Lab Corp., Northampton, MA). We have performed both heating and cooling scans during our studies. We analyzed the different domains of multicomponent melting curves using the Two State Scaled hypothesis by Launch NanoAnalyze Software. Phase transition temperatures, areas, and widths of the components were estimated with the aid of Origin nonlinear least-squares curve- and peak-fitting procedures. All thermodynamic paramaters were calculated according to published protocols.[22] DSC thermograms were plotted from "excess heat capacity" and temperature. We calculated maxima point of excess heat capacity (C_p^{max}), calorimetric enthalpies (ΔH_c), entropies

(ΔS), and full width at half maxima (FWHM). T_m is absolute phase transition temperature of sample. C_p^{max} is maxima point of excess heat capacity in main transition peak. Maximum of C_p *vs.* T curve is C_p^{max}. The Van't Hoff enthalpy is expressed by

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

Size of co-operativity unit (CU) for phase transition was determined using formula

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

Results and Discussion

Differential scanning calorimetry studies: Neat DPPC membranes exhibit main transition at ~41.3 °C apart from a pre-transition at ~35.7 °C.[24] LCA-TMA on 5 mol% doping causes progressive broadening of gel-to-liquid-crystalline phase transition and abolishes its pretransition (Figure 2a) similar to cholestrol/DPPC interactions.[25] Broadening of main acyl chain transition regularly increases with increase in doping from 5 to 50 mol%. The phase transition temperature (T_m) of main phase transition decreases with increasing doping percentage of LCA-TMA. Similarly doping of LCA-DME induces broadening of main phase transition with decrease in T_m (Figure 3a). Even 50 mol% doping of LCA-TMA or LCA-DME does not abloish T_m of phospholipid membranes, whereas 20 mol% of cholesterol based cationic lipid abolishes the DPPC phase tranistion.[25] This differential behavior of cholesterol and LCA based cationic lipids suggest that LCA-TMA and LCA-DME gel with DPPC membranes and do not abolish the motions of DPPC chains as effectively as cholesterol. This differentail behavior may be due to absence of a double bond and change in stereochemistry of ring A between cholesterol and lithocholic acid.

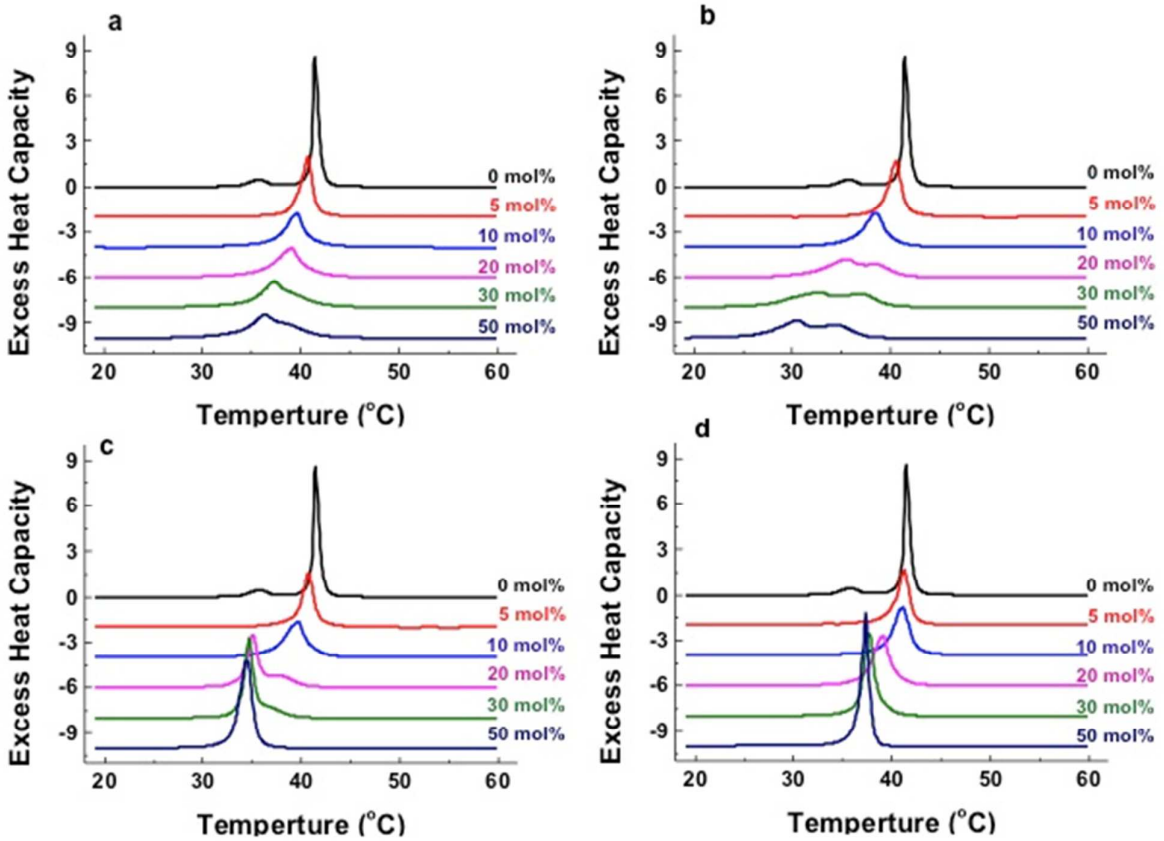

Figure 2. DSC thermograms illustrating the effect of different percentages of doping of bile acid based cationic lipids on gel to liquid-crystalline phase transition of DPPC liposomes: **a)** LCA-TMA, **b)** CDCA-TMA, **c)** DCA-TMA, **d)** CA-TMA.

Doping of CDCA-TMA and CDCA-DME induces boradening of phase tranistion as observed in case of LCA-TMA and LCA-DME doped membranes (Figure 2b, 3b). Increase in doping to 20 mol% decreases T_m of DPPC membranes, and induces phase seperation between DPPC-rich domains and cationic lipid-rich domains (Figure 2b, 3b) on further increase in doping. Presence of free hydroxyl group at 7'-position does not allow the CDCA-TMA and CDCA-DME to pack well with DPPC membranes as in case of LCA based cationic lipids. Therefore CDCA-TMA and CDCA-DME disrupts the DPPC membranes and induces phase seperation in CDCA lipid doped membranes.

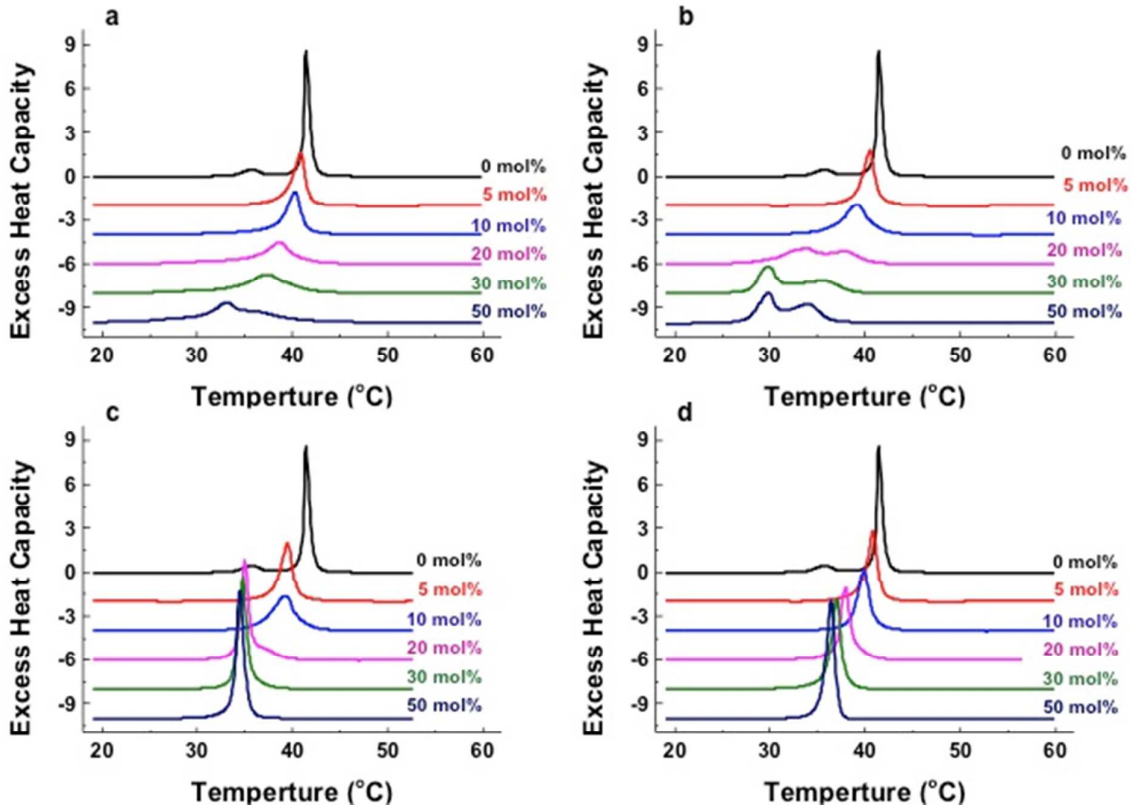

Figure 3. DSC thermograms illustrating the effect of different percentages of doping of bile acid based cationic lipids on gel to liquid-crystalline phase transition of DPPC liposomes: a) LCA-DME, b) CDCA-DME, c) DCA-DME, d) CA-DME.

We observed that intial doping (10 mol%) of DCA-TMA and DCA-DME broadens the phase transition of DPPC membranes (Figure 2c, 3c). Sharpness of phase transition increases on further doping of DCA-TMA and DCA-DME as opposed to doping of LCA and CDCA based cationic lipids. Similarly, intial doping of CA-TMA and CA-DME broadens main transition as in case of DCA based cationic lipids and further increase in doping enhances sharpness of tranistion (Figure 2d, 3d). This unexpected increase in sharpness of transition suggests that intial doping of DCA and CA cationic lipids allows distribution of cationic lipids in membranes causing disruption and broadening of transition. Further increase in doping of these lipids may allow self-assembly of

DCA or CA cationic lipids in DPPC membranes, thereby minimizing the disruptions and broadening of transition.

Effect on phase transition temperature (T_m): Doping of bile acid cationic lipids in general decreases T_m of DPPC membranes (Figure 4, Table S1, S2 (ESI)). On 50 mol% doping T_m gets lowered to ~36 ^oC and ~34 ^oC respectively for LCA-TMA and LCA-DME. Doping of CDCA-TMA and CDCA-DME drops T_m drastically and introduces two-phase transition (Table S1, S2 (SI)). Doping of 50 mol% of CDCA-TMA and CDCA-DME introduces two phase transitions with T_m ~30 ^oC and ~34.5 ^oC respectively. Therefore presence of free hydroxyl group at 7'-position in CDCA cationic lipids disrupts bile acid-DPPC packing and lowers T_m to greater extent than LCA cationic lipids.

Doping of DCA-TMA and DCA-DME lowers T_m to ~35 ^oC at 20 mol% doping and this T_m does not drop on further doping to 50 mol% (Figure 4, Table S1, S2 (ESI)). This stabilization in T_m indicates that on initial doping of 20 mol% DCA-TMA or DCA-DME molecules distribute themselves in DPPC liposomes and disrupts the membranes. Further increase in doping may bring DCA-TMA/DCA-DME molecules to form different modes of self-aggregates that stabilize the liposomes. These stable interactions does not allow further disruptions in the membranes, and does not lower the T_m further. Therefore, different positioning of free hydroxyl groups in case of CDCA and DCA lipids regulates the interactions of these cationic lipids in DPPC membranes. CA-TMA and CA-DME on 20 mol% doping lowers T_m to ~39.0 ^oC and ~38.0 ^oC respectively that does not change on further doping. CA and LCA based cationic lipids induces minimum drop in T_m (Figure 4c, 4d), whereas CDCA based cationic lipids induces maximum lowering of ~11 ^oC in T_m due to enhanced disruptions in the membranes (Table S1, S2, ESI).

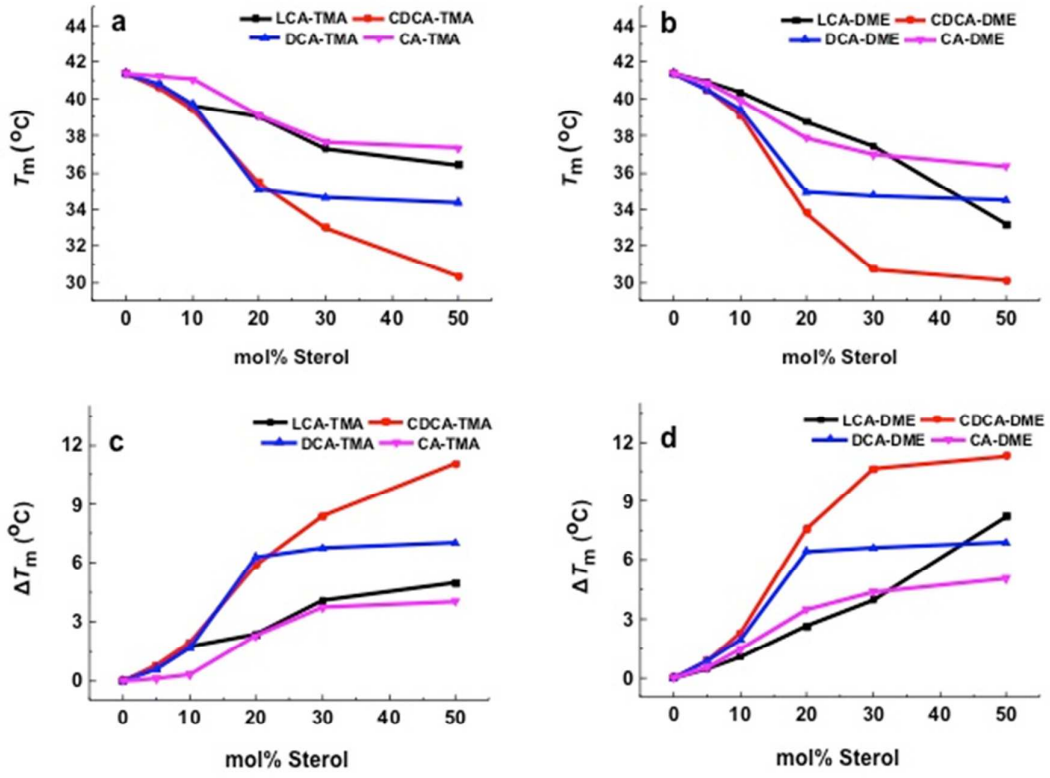

Figure 4. a, b) Effect of doping of bile acid based cationic lipids on phase transition temperature (T_m) of DPPC liposomes; **c, d)** Change in phase transition temperature (ΔT_m) of co-liposomes of DPPC with different bile acid based cationic lipids.

Effect on enthalpies and entropies of transition: We then calculacted enthalpies and entropies of transition of DPPC membranes on doping with bile acid cationic lipids as shown in Figure 5, Table S1, S2 (ESI). Doping of cholesterol and cholesterol based lipids in general decreases the enthalpies and entropies of transition of co-liposomes. The enthalpies of transition of DPPC liposomes on doping with bile acid cationic lipids in general increases with increase in doping (Figure 5a, 5b). Similarly we have observed an increase in entropies of transition from gel to fluidic phase of liposomes on increase in doping percentages of bile acid based cationic lipids (Figure 5c, 5d).

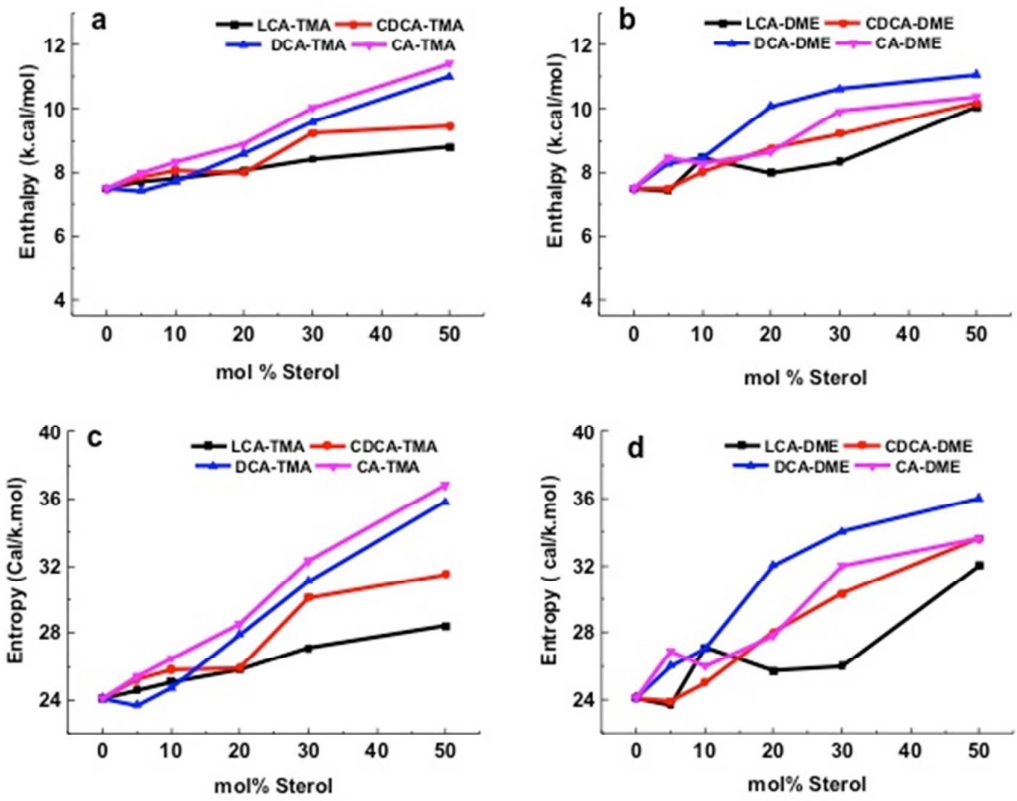

Figure 5. a, b) Effect of doping of bile acid based cationic lipids on calorimetric enthalpies of transition (ΔH_c) of DPPC liposomes; **c, d)** Effect of doping of bile acid based cationic lipids on calorimetric entropies (ΔS) of transition of DPPC liposomes.

Effect on full width at half maxima (FWHM or ΔT1/2) and Cooperativity unit (CU): DPPC membranes are composed on self-assembly of phospholipids. Co-operativity unit (CU) is cluster of self-assembled molecules undergoing phase tranistion from gel to liquid ordered phase, and depends on full width at half maxima (FWHM or $\Delta T1/2$). Increase in doping of LCA-TMA and LCA-DME lipids in DPPC membranes enhances $\Delta T_{1/2}$ (Figure 6a, 6b, Table S1, S2 (SI)) suggesting that these molecules disrupt the membrane packing like cholesterol. Doping of CDCA-TMA and CDCA-DME lipids increases $\Delta T_{1/2}$ more than LCA cationic lipids due to presence of free hydroxyl group at 7'- position. Initial doping of DCA and CA cationic lipids raises $\Delta T_{1/2}$ but further doping lowers $\Delta T_{1/2}$ (Figure 6a, 6b). These results suggest that initial doping of DCA and CA cationic lipids induces disruptions in membranes and further doping may allow these molecules to assemble themselves and therefore lowers the $\Delta T_{1/2}$. The order of increase in $\Delta T_{1/2}$ on 50 mol% doping of cationic lipids is CDCA-TMA/DME > DCA-TMA/DME > CA-TMA/DME ~ LCA-TMA/DME (Table 1, 2).

Doping of LCA-TMA and LCA-DME lipids lowers the co-operativity of DPPC membranes (Figure 6c, 6d). Similarly, doping of CDCA-TMA and CDCA-DME reduces co-operativity in DPPC lipids, as these lipids induce membrane disruptions. DCA and CA based lipids lowers co-operativity with up to 10% doping, whereas further increase in doping raises co-operativity of transition (Figure 6c, 6d). These results indicate that increase in doping may allow self-assembly of these lipids in membranes and increases the co-operativity (Table S1, S2). The order of co-operativity index at 50% doping is CA-TMA > DCA-TMA > CDCA-TMA > LCA-TMA clearly suggesting that positioning of hydroxylation on bile acids determines the co-operativity of membrane transition.

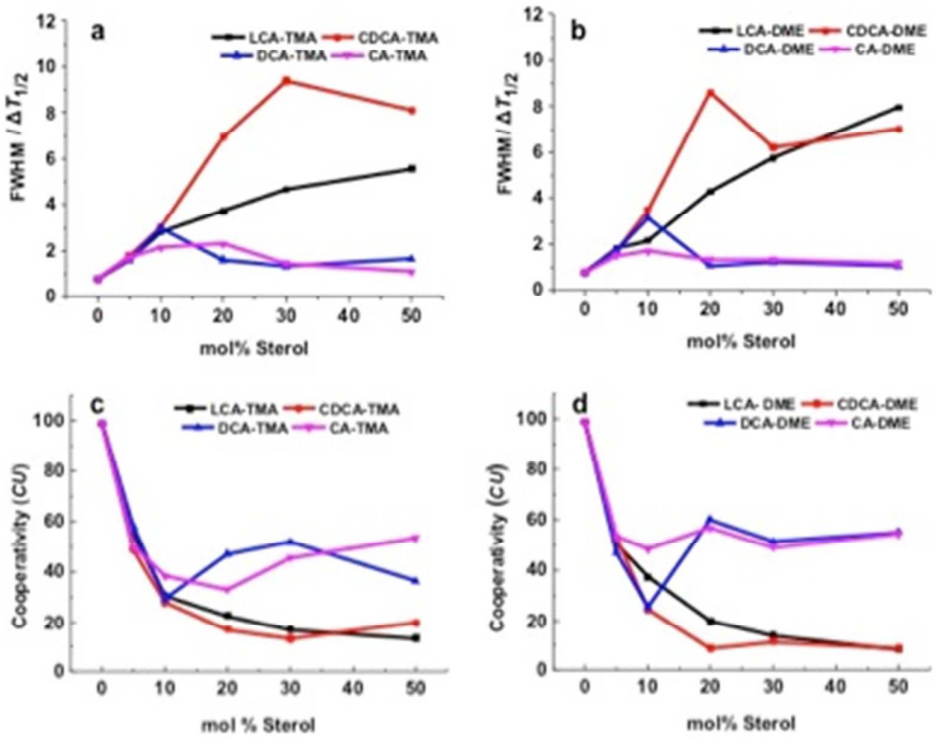

Figure 6. a, b) Effect of doping of bile acid based cationic lipids on full width half maxima (FWHM / $\Delta T_{1/2}$) of DPPC liposomes; **c, d)** Effect of doping of bile acid based cationic lipids on cooperativity (CU) of transition of DPPC liposomes.

Two-component system: We then analyzed the phase transition of DPPC-cationic lipid co-liposomes using two-phase transition model. Asymmetric DSC endotherm corresponding to main phase transition of DPPC consist of at least of two overlapping thermal events. Sharp component involves the chain-melting phase transition to sterol-poor lipid domains and broad component involves melting of sterol-rich lipid domains. We decomposed the DSC endotherms into sharp and broad components as shown in Figure 7, 8. T_m of sharp and broad component in general decreases with increase in doping of bile acid based cationic lipids. We observed maximum lowering of T_m of

sharp component at ~30 ^{o}C on doping of CDCA-TMA or CDCA-DME lipids, suggesting that free hydroxyl group at 7'-carbon of bile acid induces maximum distortions.

Sturtevant has initially reported the domain formation induced by cholesterol in DPPC membranes from the changes in shape of peak of transition.[26] Dual peaks of phase transition in bile acid lipid doped DPPC membranes indicate the formation of domains. This domain formation strongly depends on nature on the bile acid and % of doping. Doping of these cationic lipids induces domain formation in membranes even at 5 mo% of doping. Increase in doping of LCA-TMA and LCA-DME brings the DPPC lipid molecules from bile acid rich-domains to DPPC rich-domain. We observed an increase in broad peak component of transition due to cationic bile acid rich domain, and decrease in intensity of sharp DPPC-rich domain. Formation of DPPC-rich and cationic bile acid-rich domains becomes more evident on doping of CDCA cationic lipids (Figure 7, 8) as we observed two clear phase transitions on 20 mol% of doping. On higher doping, these two domains become more evident in spite of further broadening of peak. These observations suggest that enhanced doping of CDCA cationic lipids enriches these two domains. Doping of DCA and CA based cationic lipids induces domain formation up to 20 mol% of doping in DPPC liposomes. Further doping enhances the component of DPPC-enriched domain without any change in broad component. These observations clearly indicate that initial doping of DCA and CA based lipids induces domain formation, whereas further doping of these cationic lipids allow these lipid molecules to gel well within the DPPC-richer domains due to their self assembly. These liposome systems are reversible in nature, as we have observed all the scans reversible in nature including the domain formations.

Therefore, above studies showed that LCA cationic lipids are effective in quenching the acyl chain motions of DPPC lipids reflecting their lower cooperativity unit. CDCA cationic lipids induces maximum disruptions in DPPC membranes suggesting that free hydroxyl group at C_7 does not allow the packing of CDCA lipids in DPPC membranes like LCA lipids. This might be due to close

proximity of hydroxyl group to the cationic charged head group that might enhance the hydration environment around the head group resulting in more repulsions and imperfect packing. In case of DCA lipids, presence of distal C_{12} hydroxyl group from the charged head group can help in self-assembly of DCA cationic lipids in DPPC membranes. This self-assembly of DCA cationic lipids allow better packing in the membranes and therefore increases the cooperativity of membranes. Similarly, CA lipids form self-assembly in DPPC membranes like DCA lipids due to presence of C_{12} hydroxyl group that dominates the disrupting effects of C_7 hydroxyl group present in CA lipids. These results suggest that DCA and CA conjugated cationic lipids having free hydroxyl group at 12'-position induce high co-operativity on 50 mol% doping. Higher doping of DCA and CA based cationic lipids may allow these lipids to form self assembly through their concave side. This self assembly would allow the hydrophobic convex side of DCA and CA cationic lipids to interact with hydrophobic chains of DPPC lipids and increases the cooperativity among different lipid molecules. Thus distal position of hydroxyl group from the charged head group of the membranes could essentially self-assembles at higher concentrations there by increases the cooperativity of the DPPC molecule.

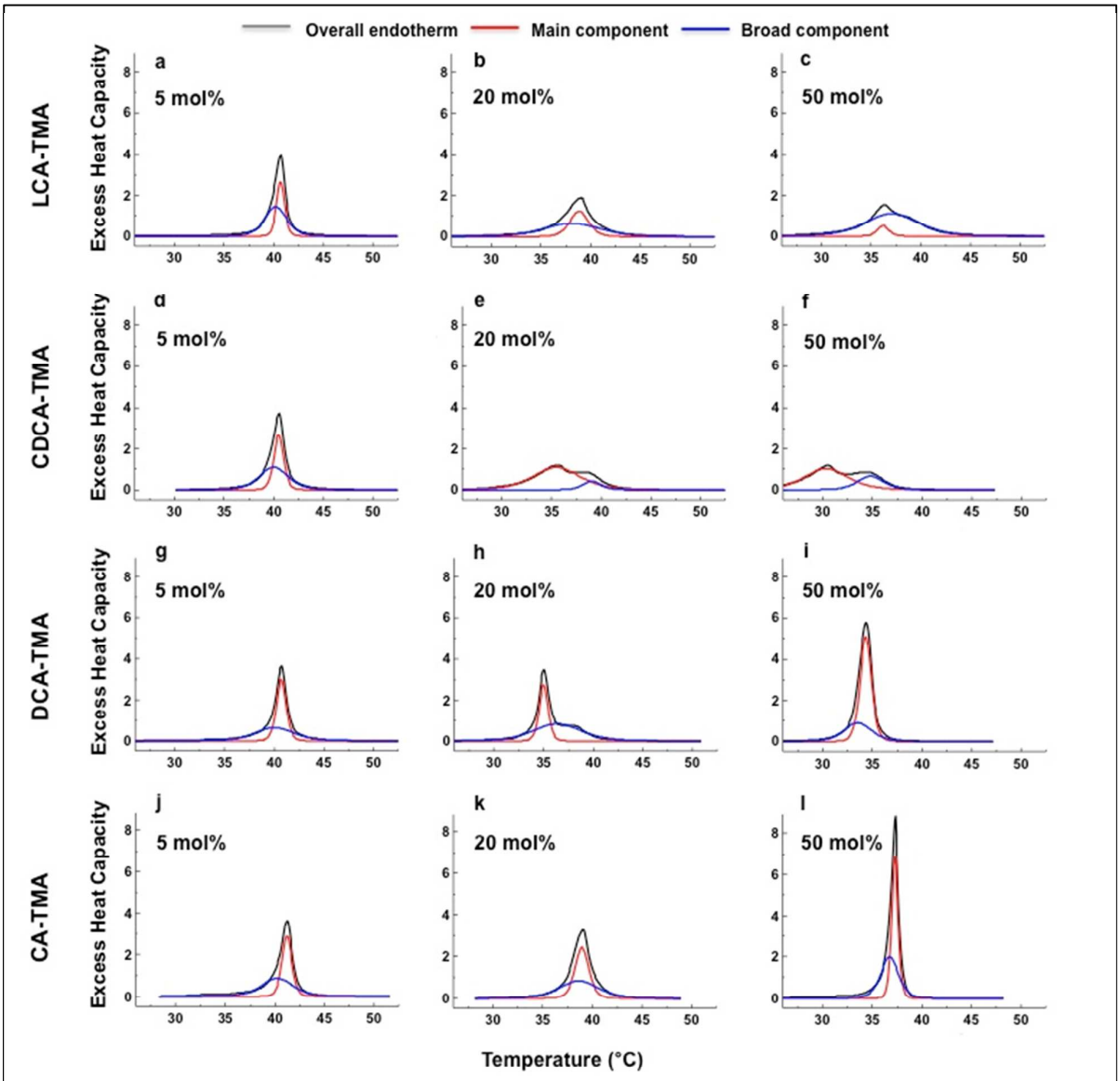

Figure 7. Decomposed endotherms of main phase transition of DPPC liposomes on incubation with 10, 20, 50 mol % of various bile acid cationic lipids. **a-c)** LCA-TMA, **d-f)** CDCA-TMA**, g-i)** DCA-TMA, **j-l)** CA-TMA.

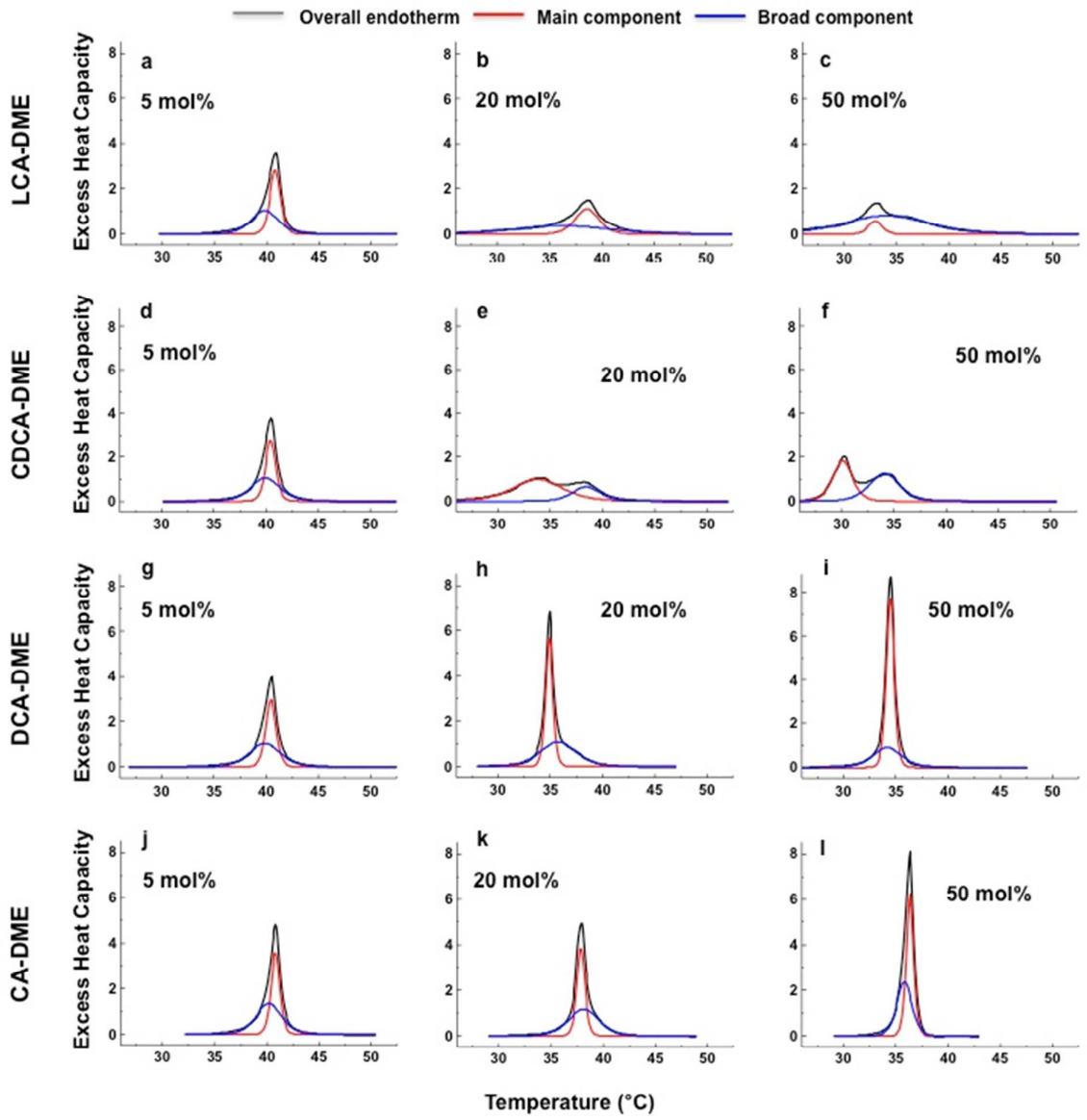

Figure 8. Decomposed endotherms of main phase transition of DPPC liposomes on incubation with 10, 20, 50 mol % of various bile acid cationic lipids. **a-c)** LCA-DME, **d-f)** CDCA-DME, **g-i)** DCA-DME, **j-l)** CA-DME.

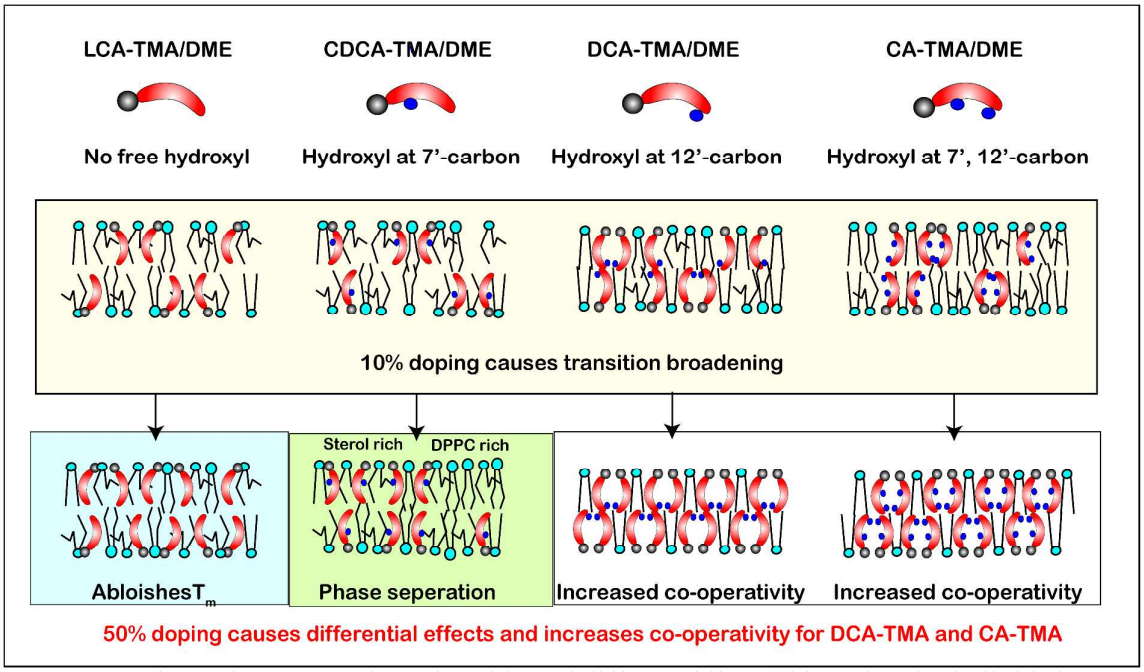

Figure 9. Schematic presentation of packing of different bile acid based cationic lipids in DPPC liposomes.

Conclusions: We presented in-depth calorimetric study of interactions of cationic bile lipids with model DPPC membranes. The interactions of cationic bile acids with model membranes revealed that phase behavior of membranes strongly depends on structure and nature of cationic bile acids. The number and positioning of free hydroxyl groups on hydrophobic core of bile acids influences the phase behavior of membranes. DCA and CA based cationic lipids showed high co-operativity on doping in DPPC membranes. CDCA lipids disrupt the DPPC membranes to maximum extent and LCA lipids gel well in membranes. Therefore number and positioning of free hydroxyl group induces differential modes of interactions of cationic bile acids with membranes irrespective of the cationic head group. These studies have suggested that inter-lipidic interactions of cationic DCA and CA bile acids on increase in doping influences the phase behavior of membranes. These new inter-lipidic interactions can influence the drug encapsulation and drug release efficacies of these liposomes that would be explored in future.

Notes and references

†Electronic Supplementary Information (ESI) (Table S1 and Table S2) available: [See DOI: 10.1039/b000000x/

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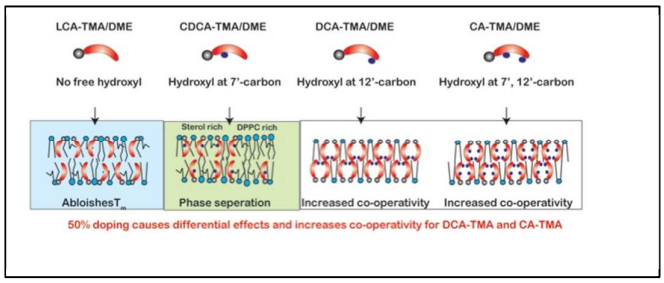

Number and Positioning of Hydroxyl Groups on Bile Acid Lipids Determine Phase Transition and Cooperativity of Membranes.