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Number of Free Hydroxyl Groups on Bile Acid Phospholipids Determines the Fluidity and Hydration of Model Membranes

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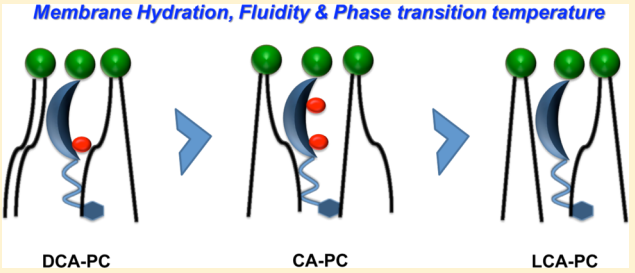
ABSTRACT: Interactions of synthetic phospholipids with model membranes determines the drug release capabilities of phospholipid vesicles at diseased sites. We performed 1,6-diphenyl-1,3,5-hexatriene (DPH)-based fluorescence anisotropy, Laurdan-based membrane hydration, and differential scanning calorimetry (DSC) studies to cognize the interactions of three bile acid phospholipids, lithocholic acid-phosphocholine (**LCA-PC**), deoxycholic acid-phosphocholine (**DCA-PC**), and cholic acid-phosphocholine (**CA-PC**) with model membranes. These studies revealed that bile acid phospholipids increases membrane fluidity in **DCA-PC** > **CA-PC** > **LCA-PC** order, indicating that induction of membrane fluidity is contingent on the number and positioning of free hydroxyl groups on bile acids. Similarly, **DCA-PC** causes maximum membrane perturbations due to the presence of a free hydroxyl group, whereas **LCA-PC** induces gel phase in membranes due to hydrophobic bile acid acyl chain interactions. These **DCA-PC**-induced membrane perturbations induce a drastic decrease in phase transition temperature (T_m) as determined by calorimetric studies, whereas doping of **LCA-PC** causes phase transition broadening without change in T_m. Doping of **CA-PC** induces membrane perturbations and membrane hydration like **DCA-PC** but sharpening of phase transition at higher doping suggests self-association of **CA-PC** molecules. Therefore these differential mode of interactions between bile acid phospholipids and model membranes would help in the future for their use in drug delivery.



INTRODUCTION

Phospholipids play a critical role in structural and functional dynamics of cell membranes. Structural diversity within phospholipids assists in functional differences like lipid-signaling cascade, protective barrier at cell surfaces, transport of small molecules, and diffusion of integral proteins.[1] Structural diversities in phospholipids is also crucial in evolutionary aspects of organisms such as archaebacterial phospholipids,[2] and Lipid A (a glycophospholipid)[3] in gram-negative bacteria. Apart from these structural and functional differences, phospholipid−membrane (lipid−lipid) interactions are crucial for bacterial and viral infections, vesicular fusion such as endocytosis and exocytosis, transport of cellular signals, import and export of cellular materials, and cellular signaling.[4]

Phospholipid-based vesicular membranes also have the capability of drug encapsulation, and their controlled release at diseased sites.[5] Naturally occurring and synthetic phospholipids have been engineered and explored for their drug delivery capabilities.[6] The interaction of these phospholipids with cellular membranes is critical for efficient transport of drugs in intracellular compartments.[7] Szoka et al. has recently explored the interactions of steroidal based phospholipids with membranes and its drug delivery.[8,9] Their studies revealed that doxorubicin encapsulated steroidal phospholipids are more efficient than natural phospholipid based commercial liposomal formulations.

Therefore design of new steroidal phospholipids, their interactions with membranes, and drug delivery propensities is key for efficient drug delivery vehicles. Physical characteristics of these phospholipids like hydrophobicity, hydrophilicity, and charge regulate their communications with membranes.[10] Phospholipid mediated intracellular drug delivery entangles electrostatic binding at the membrane surface followed by hydrophobic interactions with lipid acyl chains.[11] These communications can alter the lipid arrangement and perturb the membranes leading to delivery of drug molecules. Therefore, biophysical insights into interactions of synthetic phospholipids with models membranes are imperative for exploiting their future use in drug delivery.

Bile acids present a class of sterol based facial amphiphiles that offers structural advantages by virtue of its hydroxylated concave structure and a carboxylic acid at C_{24} position.[12] Supramolecular aggregates of bile acids and bile salts have prime physiological importance in solubilization, digestion, and absorption of dietary fat through intestinal wall.[13,14] Interactions of bile acids with membranes is crucial for fat solubilization, its absorption, and toxicity in colon epithelium. Mishra and co-workers have shown that bile salts at submiceller concentrations induce hydration of dipalmitoylphosphatidylcholine (DPPC) and Dimyristoylphosphatidylcholine (DMPC) unilamellar vesicles.[16] Bile acids can also serve as a carrier to deliver bioactive molecules[15] and have been explored for

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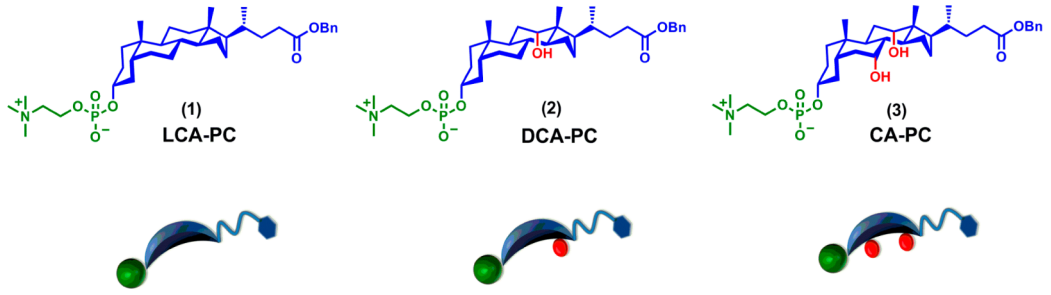

Figure 1. Molecular structures and schematic presentation of lithocholic acid-, deoxycholic acid-, and cholic acid-based phospholipids **LCA-PC**, **DCA-PC**, and **CA-PC**.

efficient delivery of anticancer drugs at tumor sites.[17] Therefore it is essential to study the interactions of bile acid based synthetic lipids with model membranes.

In this manuscript, we used three bile acid phospholipids lithocholic acid-phosphocholine (**LCA-PC**), deoxycholic acid-phosphocholine (**DCA-PC**), and cholic acid-phosphocholine (**CA-PC**) (Figure 1). These phospholipids encompass conjugation of phosphocholine headgroup to 3′-hydroxyl group of lithocholic acid (**LCA**), deoxycholic acid (**DCA**), and cholic acid (**CA**) (Figure 1). We studied interactions of these phospholipids with model membranes using diphenyl hexatriene (DPH)-based steady state fluorescence anisotropy, Laurdan based hydration studies, and in depth differential scanning calorimetry (DSC) studies. These studies have revealed that the presence of phosphocholine head groups, the number of free hydroxyl groups, and their positioning dramatically impacts interactions of bile acid phospholipids with model membranes.

EXPERIMENTAL SECTION

Materials. All chemicals used in the studies are of ACS grade. DPH and Laurdan were purchased from Sigma-Aldrich. DPPC was purchased from Avanti Polar Lipids.

Liposome Formation.[18] Desired amounts of DPPC and bile acid phospholipids were taken in round-bottom Wheaton glass vials in chloroform. Thin films were made under dry argon gas, and films 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.

Fluorescence Anisotropy Studies.[19] Fluorescence anisotropy studies were performed on unilamellar DPPC vesicles doped with different percentages of bile acid phospholipids and DPH (DPPC:DPH ratio 100:1). Steady state DPH anisotropy measurements were done in a 96-well plate using fluorescence anisotropy protocol in a Molecular Devices M5 instrument with λ_{ex} at 350 nm, and λ_{em} of 452 nm. Effect of temperature on anisotropy was studied by measuring fluorescence from 23 to 55 °C. Samples were equilibrated for at least 10 min after each temperature change. Steady state fluorescence anisotropy (r_{ss}) was then calculated using the following eq (I)

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

Where $I_{\parallel}$ and $I_{\perp}$ are emission intensity excited with parallel-polarized light and measured with an 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 parallel and perpendicular polarized emission intensities after excitation with perpendicularly polarized light. Sigmoidal curve fitting of experimental points were performed to deduce phase transition temperature using OriginPro 8.5.1 software. Phase transition temperature due to gel-to-liquid crystalline transition was calculated from midpoint first derivatives of temperatures versus anisotropy curves.

Laurdan-Based Hydration Studies.[20] Unilamellar mixed vesicles comprising DPPC and bile acid phospholipids along with laurdan were prepared in a way similar to that described above. The generalized polarization experiments were performed in a 96-well plate in a Molecular Devices M5 instrument. The fluorescence experiments were performed using λ_{ex} of 350 nm, and end point emissions were recorded at λ_{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 10 min after each temperature change. The generalized polarization (GP) was calculated using eq II

$$GP = (I_{440} - I_{490})/(I_{440} + I_{490}) \quad \text{(II)}$$

Differential Scanning Calorimetry.[21] DSC studies were performed on Nano DSC instrument, TA Instruments, USA using DPPC liposomes or coliposomes of DPPC with bile acid phospholipids. All reference and sample suspensions were degassed prior to being placed in DSC to minimize the possibility of gas bubble formation during the run. After baseline runs, we run liposomal samples by filling the sample cell with degassed liposomal suspensions and the reference cell with Milli Q water. DSC measurements were carried out in the temperature range of 15−60 °C, with heating and cooling scan rates maintained at 1 °C/min for all experiments. DSC thermograms for liposomal suspensions of DPPC and coliposomes were obtained by subtracting the appropriate baseline from the sample thermogram using NanoAnalyze software provided by the manufacturer. Calorimetric enthalpies (ΔH_c) and entropies (ΔS) were computed using NanoAnalyze software. Maximum point of excess heat capacity (C_p^{max}), and full width at half maxima (FWHM) were calculated using OriginPro 8.5.1 software by nonlinear curve fitting with gauss function. The size of the cooperativity unit (CU) for phase transition of each lipid was determined using formula III.[22]

$$CU = \Delta H_{vH}/\Delta H_c \quad \text{(III)}$$

where ΔH_{vH} is van't Hoff enthalpy, and ΔH_c is calorimetric enthalpy for a lipid gel to liquid crystalline phase assuming a two-state transition.

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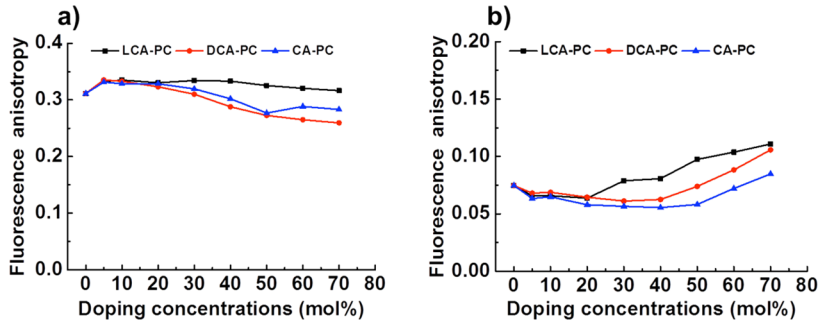

Figure 2. (a) Changes in DPH anisotropy of DPPC membranes in gel phase at 23 °C on doping of different mol % of bile acid phospholipids **LCA-PC**, **DCA-PC**, and **CA-PC**. (b) Changes in DPH anisotropy of DPPC membranes in liquid crystalline (LC) phase at 55 °C on doping of different mol % of bile acid phospholipids **LCA-PC**, **DCA-PC**, and **CA-PC**.

The process of transition becomes affected by broadening of the transition and is approximately expressed in terms of the van't Hoff equation. The presence of various solutes/other molecules contributes to the broadening of the transition. The two contributions to broadening of the transition, (i) van't Hoff broadening and (ii) solute broadening, are additive.[23,24] Hence these two contributions were accounted for in the van't Hoff enthalpy calculations by Sturtevant.[25] In multicomponent heterogeneous systems like bile acid phospholipids-DPPC, added solute (bile acid phospholipids) will impact the thermodynamic properties of DPPC; therefore the addition of mole fraction (X) would give an approximation of van't Hoff enthalpy using eq IV.[24,26]

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

To understand the different domain formations in thermograms of multicomponent systems, we deconvoluted the DSC thermograms using the in-built scaled two-state transition model provided in the NanoAnalyze software. Deconvolution of single or multi-transition, multicomponent thermograms by the two-state model results in a linear combination of multiple, independent, Gaussian two-state transitions. This deconvolution modeling algorithm uses a nonlinear least-squares method where modeled thermograms with an overlapping residual of at least 95% confidence limit will be considered a good fitting.[27,28] The equation used in the deconvolution algorithm to quantify C_p is

$$C_p = A_w(\Delta H)^2/(RT^2)\exp[\Delta H(-T_m + T)/(T_m TR)]/(1 + K)^2$$

where $K = \exp[-\Delta H(1 - T/T_m)/(RT)]$.

T is the temperature in Kelvin, R is the gas constant, T_m is the temperature of the transition peak maximum, ΔH is the enthalpy change for the transition with an assumed molecular weight, A_w is a weighting factor proportional to the amount of material in the transition, and C_p is excess heat capacity. The A_w fit parameter is a scaling factor that takes into account any errors in the molecular weight or active concentration of macromolecule. The A_w factor corresponds to the relative abundance of materials undergoing the transition. In order to reduce the standard deviation between the sum of decomposed individual transition peaks and the actual thermogram, a scaling factor (A_w) was introduced in the two-state model, called the scaled two-state model, that is a in-built algorithm in the NanoAnalyze software.[28−30]

RESULTS AND DISCUSSION

Fluorescence Anisotropy Studies. We studied the effect of phospholipid doping on the motion of acyl chains in DPPC membranes using DPH as a probe.[26] Doping of 5 mol % of **LCA-PC** in DPPC membranes in gel phase increases DPH anisotropy, which drops with further increase in percentage of doping (Figure 2a, Table 1). In gel phase, **LCA-PC**-doped DPPC membranes showed higher anisotropy as compared to neat DPPC membranes, indicating that integration of **LCA-PC** causes more ordering and rigidification of DPPC membranes. **LCA-PC** molecules due to their hydrophobic nature do not disturb packing of DPPC membranes and induce gel phase. In gel phase, 5−20 mol % doping of **DCA-PC** increases

Table 1. Changes in Fluorescence Properties of DPH in DPPC Liposomes on Doping of Different Percentages of Bile Acid Phospholipids LCA-PC, DCA-PC, and CA-PC

compound	mol %	r_{ss} at 23 °C	r_{ss} at 55 °C	T_m (°C)
DPPC		0.311	0.075	42.5
LCA-PC	5	0.332	0.066	42.3
	10	0.335	0.066	42.3
	20	0.330	0.064	41.0
	30	0.334	0.079	41.3
	40	0.333	0.081	41.6
	50	0.325	0.097	41.0
	60	0.321	0.104	39.6
	70	0.316	0.111	37.0
DCA-PC	5	0.335	0.068	42.6
	10	0.333	0.069	42.3
	20	0.323	0.064	38.6
	30	0.310	0.061	35.9
	40	0.288	0.062	33.6
	50	0.273	0.074	31.2
	60	0.265	0.088	-
	70	0.259	0.106	-
CA-PC	5	0.331	0.063	41.8
	10	0.328	0.065	41.3
	20	0.328	0.058	38.7
	30	0.319	0.057	35.9
	40	0.302	0.055	33.0
	50	0.277	0.058	31.3
	60	0.288	0.072	31.8
	70	0.283	0.085	31.8

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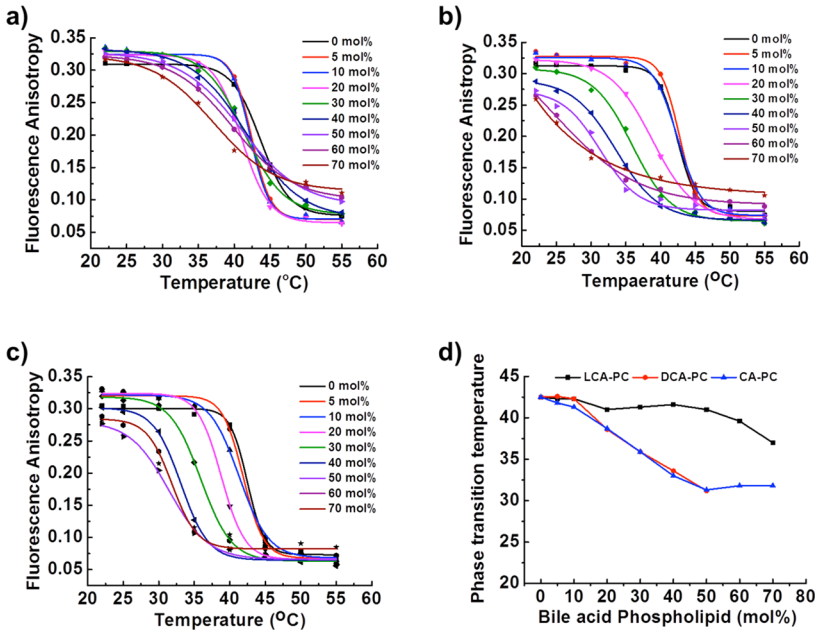

Figure 3. (a–c) Effect of temperature on DPH fluorescence anisotropy of DPPC membranes on doping of different percentages of bile acid phospholipids: (a) **LCA-PC**, (b) **DCA-PC**, (c) **CA-PC**. (d) Effect of doping of different percentages of bile acid phospholipids **LCA-PC**, **DCA-PC**, and **CA-PC** on phase transition temperature (T_m) by DPH-based anisotropy studies.

anisotropy of DPPC membranes, suggesting rigidification of membranes. More than 20 mol % of **DCA-PC** doping fluidizes the membranes with maximum fluidity at 70% doping (Figure 2a, Table 1), whereas **LCA-PC** doping could not fluidize DPPC membranes even at 70 mol % doping in gel phase. More than 40 mol % doping of **CA-PC** lipids in DPPC membranes is required to fluidize the membranes (Figure 2a, Table 1). Therefore, the order of induction of membrane fluidity in gel phase of DPPC membranes is **DCA-PC** > **CA-PC** > **LCA-PC**. In gel phase, **DCA-PC** and **CA-PC** result in maximum disturbances in packing of DPPC membranes, causing more fluidity as compared to **LCA-PC** (Figure 2a). These results suggest that introduction of free hydroxyl groups on bile acid phospholipids **DCA-PC** and **CA-PC** disturb hydrophobic interactions and packing between bile acids and acyl chains of lipids, whereas **LCA-PC** lipids without any free hydroxyl group helps in hydrophobic interactions between bile acids and DPPC lipid chains, inducing rigidification of membranes.

Doping of phospholipids induces rigidification of DPPC membranes in their fluidic/liquid crystalline (LC) phase, and this induction is contingent on the number of free hydroxyl groups. Doping of 30 mol % and 40 mol % is required for **LCA-PC** and **DCA-PC** to rigidify the membranes, respectively (Figure 2b), whereas **CA-PC** induces minor rigidification of the fluidic phase only at 60–70 mol % doping. Therefore, the order of induction of membrane rigidity by bile acid phospholipids in the LC phase is **LCA-PC** > **DCA-PC** > **CA-PC**. These results exposed that **LCA-PC** having no free hydroxyl group induces gel phase and rigidifies the membranes in gel and LC phase due to the capability of hydrophobic bile acid long chain interactions. **DCA-PC** and **CA-PC** having free hydroxyl groups are not able to induce gel phase in DPPC membranes in gel and LC phase, and therefore do not rigidify membranes as free hydroxyl groups perturb the hydrophobic interactions.

Temperature-dependent anisotropy studies showed that doping of 50 mol % of **LCA-PC** does not change the phase transition temperature (T_m) of DPPC membranes (Figure 3a). 70 mol % doping of **LCA-PC** reduces T_m to 37 °C (Table 1) without abolishing its phase transition. These results indicate that **LCA-PC** forms more ordered structural assemblies in DPPC membranes even up to 70 mol % doping, as opposed to cholesterol and cholesterol-based lipids that abolish phase transition of membranes even on 30 mol % doping.[31] Therefore, stereochemical differences in lithocholic acid and cholesterol may allow **LCA-PC** molecules to induce gel with DPPC lipids, and do not cause disordering of membranes. Doping of **DCA-PC** induces a systematic decrease of T_m to 30 °C at 50 mol % doping (Figure 3b, Table 1) and its abolishment on further doping. **DCA-PC** lipids due to the free hydroxyl group induces perturbations in packing of acyl chains and causes lowering of phase transition as opposed to **LCA-PC**-doped DPPC membranes where hydrophobic interactions due to the absence of free hydroxyl group does not allow abolishment of T_m. **CA-PC** on 50 mol % doping lowers T_m to ~31.5 °C, which does not get abolished even on 70 mol % doping (Figure 3c, Table 1) unlike **DCA-PC** doping. These results indicate that introduction of two free hydroxyl group as in the case of **CA-PC** lowers T_m at initial doping concentrations, but higher **CA-PC** doping induces different membrane organization that does not allow abolishing of T_m, as observed in the case of **DCA-PC**-doped membranes.

Bile acid phospholipid integration in DPPC membranes induces decrease of T_m in **DCA-PC** > **CA-PC** > **LCA-PC** order (Figure 3d). Therefore hydrophobicity and the presence of hydroxyl groups on the concave surface shows a prompt influence on the fluidity and phase transition temperature of DPPC model membranes. As membrane fluidity strongly depends on the percentage of doping, hydrophobicity of bile acid, and number of free hydroxyl groups on bile acid surface, these conjugates can be suitable candidates for encapsulation of drugs in liposomal carriers for drug delivery.

Laurdan-Based Hydration Studies. In gel phase, doping of **LCA-PC** in DPPC membranes does not cause any change in GP of laurdan, suggesting that **LCA-PC** molecules do not

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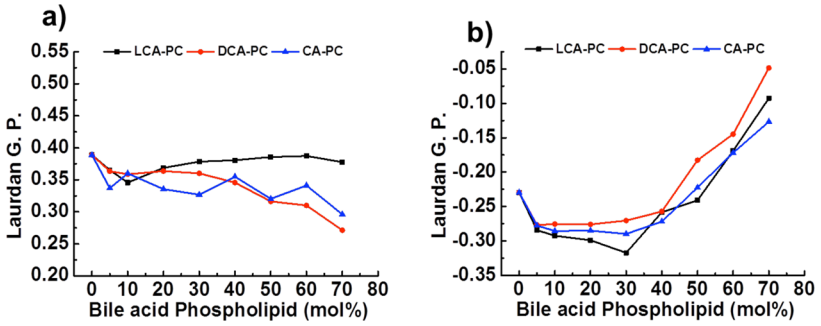

Figure 4. (a) Changes in GP of laurdan in DPPC membranes in gel phase at 23 °C on doping of different mol % of bile acid phospholipids **LCA-PC**, **DCA-PC**, and **CA-PC**. (b) Changes in GP of laurdan of DPPC membranes in LC phase at 55 °C on doping of different mol % of bile acid phospholipids **LCA-PC**, **DCA-PC**, and **CA-PC**.

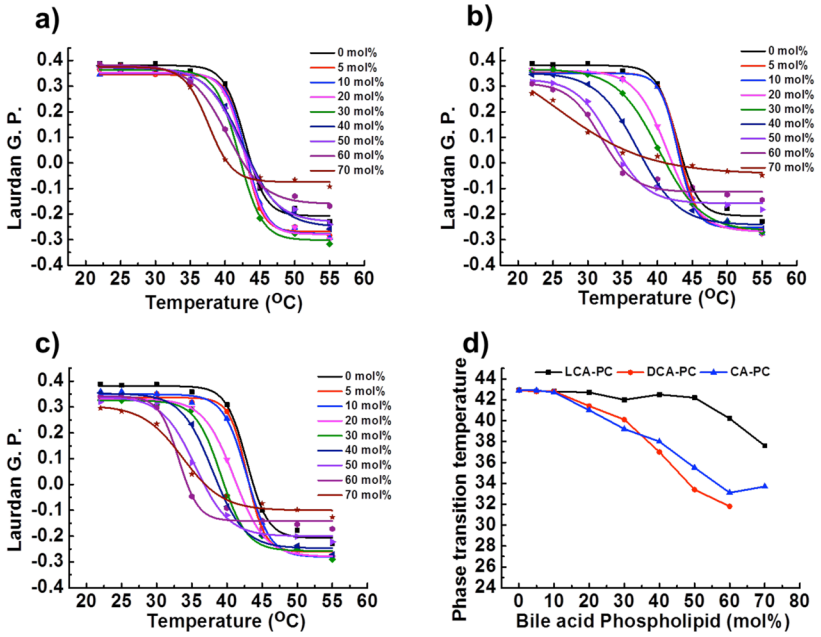

Figure 5. (a–c) Effect of temperature on laurdan GP of DPPC membranes on doping of different percentages of bile acid phospholipids: (a) **LCA-PC**, (b) **DCA-PC**, (c) **CA-PC**. (d) Effect of doping of different percentages of bile acid phospholipids **LCA-PC**, **DCA-PC**, and **CA-PC** on phase transition temperature (T_m) by laurdan-based membrane hydration studies.

change the membrane hydration in gel phase (Figure 4a). Doping of **DCA-PC** induces the increase in magnitude of dipolar relaxation of laurdan by enhanced surface water content, thereby inducing a progressive decrease in GP value of laurdan in gel phase. Similarly, **CA-PC** doping also displayed a lower GP value with increase in concentration (Figure 4a), implying systematic rise in magnitude of membrane hydration. The presence of free hydroxyl groups and asymmetric interactions in lipophilic regions of liposomes in **DCA-PC**- and **CA-PC**-doped membranes triggers resultant rise in surface hydration in gel phase. In LC phase, doping of up to 40% of bile acid phospholipids diminishes the GP value, causing more surface hydration (Figure 4b), whereas more than 40% of bile acid phospholipids escalate the GP value, indicating dehydration of membranes.

Rise in temperature in general decreases GP values, indicating the enhanced hydration at the membrane surface. Temperature-based studies showed that **LCA-PC** does not cause any change in T_m on 50% doping, and minor change in T_m of ∼5 °C was observed on 60–70% doping. These observations are consistent with anisotropy studies indicating the smooth unperturbed packing of **LCA-PC** in membranes (Figure 5a). **DCA-PC**, on the other hand, lowers the T_m of membranes and abolishes its T_m at 70% doping, indicating that **DCA-PC** fluidizes the membranes and does not allow the condensed packing like **LCA-PC** doping (Figure 5b). Similarly, doping of **CA-PC** decreases the T_m by inducing distortions in membranes (Figure 5c). The order of membrane disruptions as observed by laurdan studies is **DCA-PC** > **CA-PC** > **LCA-PC**, similar to observations in anisotropy studies. Therefore anisotropy and hydration studies conclude that hydrophobic **LCA-PC** lipids induce gel phase with DPPC membranes causing less perturbations and change in hydration. Introduction of free hydroxyl group in the case of **DCA-PC** causes membrane perturbations in hydrophobic bilayers, leading to more hydration and lowering of T_m. **CA-PC** possessing two free hydroxyl groups enhances membrane hydration but causes less perturbations as compared to **DCA-PC**, which may be due to differential molecular packing of **CA-PC** in membranes.

Differential Scanning Calorimetry. To understand the thermodynamics of interactions, we performed DSC studies of DPPC liposomes doped with bile acid phospholipids. Pure

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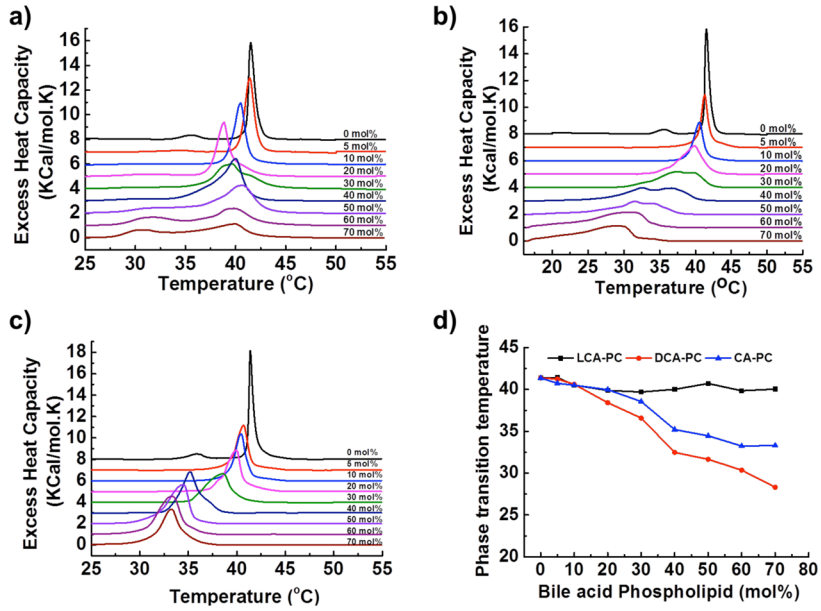

Figure 6. (a–c) Differential scanning calorimetric thermograms of neat DPPC liposomes and on doping with different percentages of bile acid phospholipids: (a) **LCA-PC**, (b) **DCA-PC**, (c) **CA-PC**. (d) Effect of doping of different percentages of bile acid phospholipids **LCA-PC**, **DCA-PC**, and **CA-PC** on phase transition temperature (T_m) as observed by DSC studies.

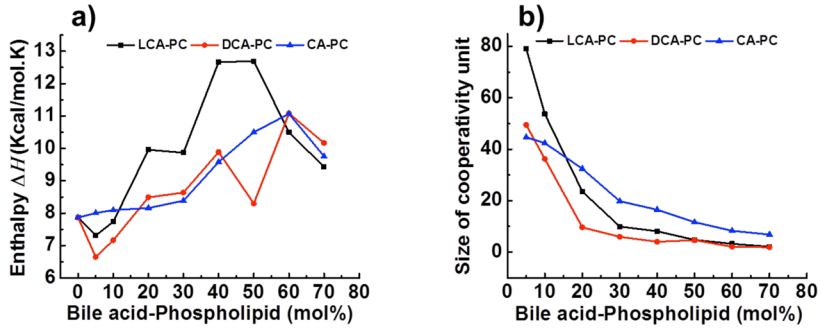

Figure 7. Effect of incorporation of different percentages of bile acid phospholipids **LCA-PC**, **DCA-PC**, and **CA-PC** on calorimetric enthalpy of coliposomes (a) and on co-operativity size (b).

DPPC membranes exhibit two endothermic peaks including a pretransition peak at ~35.7 °C and a main transition peak at 41.3 °C. **LCA-PC** completely abolishes pretransition peak and broadens main phase transition on 5 mol % doping (Figure 6a). Increasing the doping % of **LCA-PC** in DPPC membranes causes progressive broadening of the main phase transition peak without lowering the T_m of membranes (Figure 6d). **LCA-PC** causes only 1 °C change in T_m value even on 70 mol % doping, indicating favorable hydrophobic interactions between the concave surface of bile acids and acyl chains of DPPC, which does not allow lowering of T_m.

Similarly **DCA-PC** doping abolishes the pretransition peak and broadens the main transition peak at 5 mol %, whereas further doping of **DCA-PC** decreases T_m unlike **LCA-PC** (Figure 6b,d). **DCA-PC** on 30 mol % doping lowers T_m by 5 °C and T_m gets lowered down to 28.3 °C with 70 mol % doping. This radical decrease in T_m suggests the disruption of membrane organization as **DCA-PC** with one free hydroxyl group perturbs hydrophobic interactions leading to lowering of T_m.

On the other hand, doping of **CA-PC** having two hydroxyl groups in DPPC membranes decreases T_m to a lesser extent as compared to doping of **DCA-PC**. We observed only ~5.0 °C decrease in T_m after 40% doping, and further doping of 70 mol % decreases T_m only by 2 °C (Figure c,d). Therefore the order of lowering of T_m on doping is **DCA-PC** > **CA-PC** > **LCA-PC**, which is consistent with the order of their effect on membrane fluidity observed by DPH-based anisotropy studies. Therefore DSC studies suggest that the presence of two free hydroxyl groups in **CA-PC** does not disrupt membranes and lower T_m to the same extent as **DCA-PC** with one free hydroxyl group, as introduction of two free hydroxyl group may allow **CA-PC** molecules to form self-aggregates in membranes and allow some favorable hydrophobic interactions between bile acids and DPPC lipids.

Calorimetric enthalpy indicates the actual heat change associated with the transition per mole of the system. The presence of other lipids, rafts, or molecules that interact in hydrophobic regions of membranes broadens the main transition. Such broadening is usually expressed in terms of the van't Hoff equation. van't Hoff enthalpy represents the amount of heat required for each cooperative unit that undergoes phase transition. For a first-order two-state transition $\Delta H_{vH} = \Delta H_{cal}$. A non-two-state transition involves either one or several intermediate stages where $\Delta H_{vH} < \Delta H_{cal}$ or intermolecular cooperation when $\Delta H_{vH} > \Delta H_{cal}$.[22,27] Increase

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Table 2. Thermodynamic Characterization of DPPC and Its Coliposomes with Bile Acid Phospholipids Determined by DSC

compound	mol %[a]	T_m[b] (°C)	ΔH_c (kcal/molK)	ΔS (cal/mol K)	ΔH_{vH} (kcal/molK)	CU[c]	FWHM[d]	C_p^{max}[e]
DPPC		41.3	7.87	25.00			0.61	8.74
LCA-PC	5	41.4	7.31	23.20	578.49	79.15	1.17	5.67
	10	40.5	7.74	24.70	415.64	53.70	1.46	4.58
	20	39.8	9.96	31.80	234.63	23.55	1.83	3.76
	30	39.7	9.87	31.50	98.06	9.94	4.27	1.78
	40	40.0	12.67	40.50	103.14	8.14	3.42	2.80
	50	40.7	12.69	40.40	60.05	4.73	4.20	1.95
	60	39.8	10.50	33.60	34.03	3.24	4.36	1.15
	70	40.0	9.43	30.10	19.80	2.10	12.34	0.80
DCA-PC	5	41.3	6.65	21.10	328.49	49.43	1.53	2.93
	10	41.0	7.17	22.80	259.55	36.21	1.77	2.64
	20	39.9	8.49	27.10	81.66	9.61	4.85	1.12
	30	37.6	8.64	27.80	51.29	5.94	7.41	0.83
	40	32.7	9.89	32.30	39.79	4.02	8.35	0.88
	50	31.7	8.30	27.20	38.42	4.63	7.81	0.87
	60	30.5	11.08	36.50	23.47	2.12	8.64	0.89
	70	28.7	10.17	33.70	19.39	1.91	7.20	0.91
CA-PC	5	40.7	8.01	25.50	357.78	44.67	1.51	3.86
	10	40.5	8.10	25.80	343.41	42.40	1.50	3.96
	20	39.9	8.16	26.10	264.48	32.42	2.02	3.47
	30	38.5	8.39	26.90	166.10	19.80	3.02	2.58
	40	35.2	9.58	31.10	157.67	16.45	2.61	3.34
	50	34.4	10.50	34.10	122.22	11.64	2.61	3.42
	60	33.2	11.07	36.10	91.74	8.29	2.78	3.41
	70	33.31	9.76	31.80	66.66	6.83	2.70	2.91

[a][DPPC] = 0.5 mg/mL. [b]Accuracy in T_m was ±1.0 °C. [c]Size of co-operativity unit. [d]Full width at half-maximum. [e]Maximum point of specific heat capacity.

in enthalpies in the case of **DCA-PC**- and **CA-PC**-doped DPPC membranes is associated with broadening of gel-to-liquid phase transitions as well as the sum of interaction energies between DPPC and bile acid phospholipids (Figure 7a and Table 2). **LCA-PC** shows a complex trend of enthalpies and entropies of transition. The complex trend observed with **LCA-PC** is evident as it induces fewer changes in the phase transition temperature of coliposomes and is less effective in quenching the order of DPPC acyl chains. We calculated van't Hoff enthalpies from C_p^{max}, calorimetric enthalpies, and mole fractions of doped bile acid phospholipids. Incorporation of increasing concentrations of bile acid phospholipids caused progressive decrease in van't Hoff enthalpies (Table 2). In the case of **CA-PC**, van't Hoff enthalpies also decreased less rapidly on increase in doping. van't Hoff enthalpy on 70 mol % doping of **CA-PC** was nearly 3-fold more than doping of **LCA-PC** and **DCA-PC**. van't Hoff enthalpies in the case of lipid bilayers were much larger than calorimetric enthalpies due to intermolecular cooperation.[23]

Doping of bile acid phospholipids **LCA-PC** and **DCA-PC** in DPPC membranes increases the FWHM ($\Delta T_{1/2}$) and reduces height of transition peak (Table 2). **LCA-PC** and **DCA-PC** induces disordered domains in DPPC membranes and quenches acyl chain interactions. Quenching of acyl chain motions of DPPC lipids in **LCA-PC**- and **DCA-PC**-doped DPPC membranes lowers the co-operativity of system (Figure 7b, Table 2). Doping of **CA-PC** lipid in DPPC membranes also induces rise in $\Delta T_{1/2}$ up to 30 mol % doping, whereas further doping of **CA-PC** does not enhance $\Delta T_{1/2}$. Doping up to 30 mol % of **CA-PC** in DPPC membranes allows **CA-PC** molecules to disrupt acyl chains interactions, and therefore lowers co-operativity (Figure 7b, Table 2). Further integration of **CA-PC** does not enhance $\Delta T_{1/2}$, and therefore does not drop the co-operativity of **CA-PC**-doped DPPC membranes unlike **LCA-PC**- and **DCA-PC**-doped DPPC membranes (Figure 7b, Table 2). These higher co-operativity of **CA-PC**-doped DPPC membranes as compared to **LCA-PC**- and **DCA-PC**-doped membranes indicate that **CA-PC** molecules may associate among themselves to form ordered microdomains in membranes. These intermolecular associations of **CA-PC** can avoid unfavorable interactions between hydrophilic concave sides of **CA-PC** with hydrophobic acyl chains of DPPC membranes. Therefore a balance between hydrophobicity and hydrophilicity of these facial amphiphiles are critical in controlling fluidity and phase transitions of membranes.

We deconvoluted DSC endotherms into sharp and broad components as the main phase transition peaks of coliposomes of phospholipids and other lipids/compounds are composed of the sum of superimposed two-component peaks (Figure 8).[27] **LCA-PC** on 40 mol % doping broadens the phase transition with two distinct maximas, indicating the existence of different lipid-rich domains (Figure 8). **LCA-PC** with a phosphocholine headgroup integrates with the glycerophoscholine region of DPPC membranes, and the concave steroidal part of bile acid establishes van der Waal's associations with the hydrophobic acyl chains of DPPC lipids. Therefore doping of **LCA-PC** broadens the transition, eventually leading to the formation of different domains in coliposomes. Doping of 70 mol % induces

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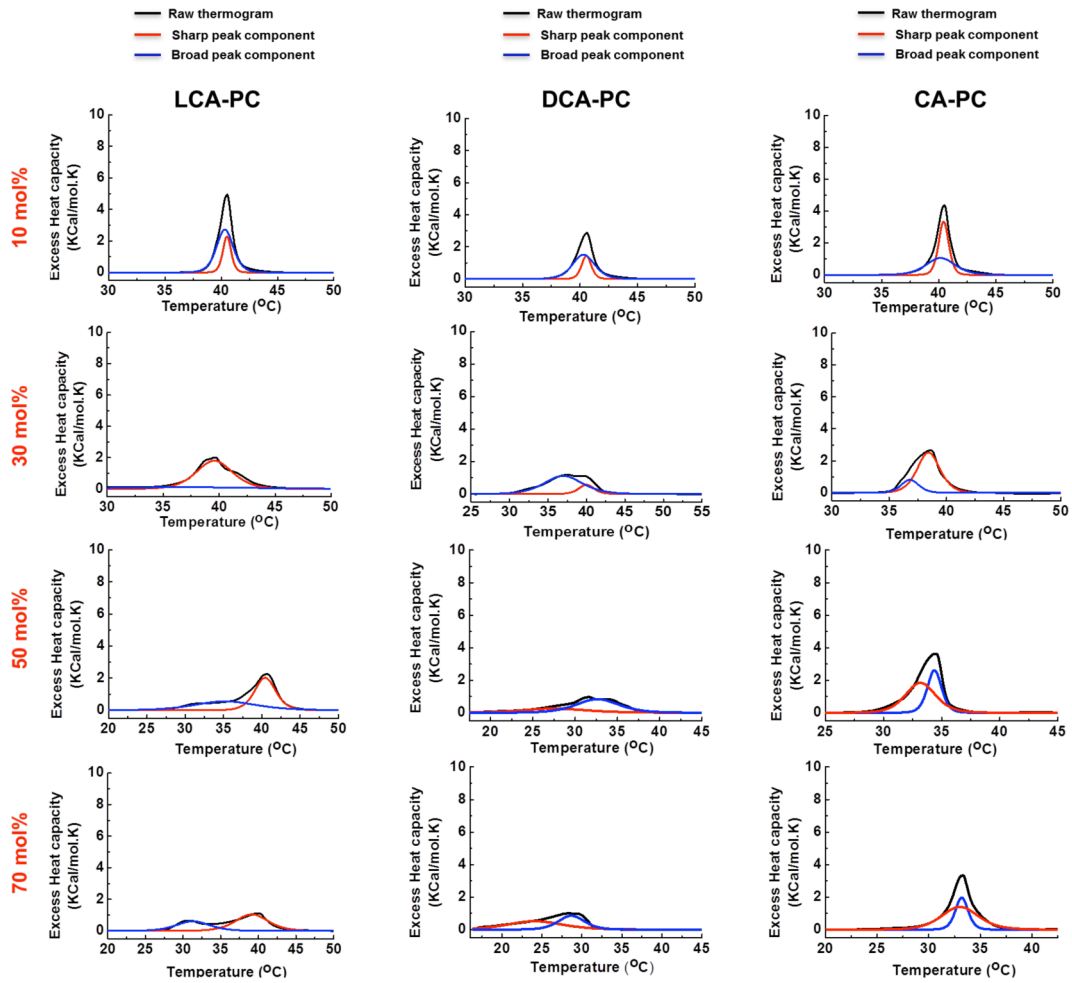

Figure 8. Raw thermogram and two-component analysis plots of DPPC liposomes doped with different percentages of bile acid phospholipids. Raw thermograms were decomposed into two components, one of which is a sharp component consisting of a DPPC-rich domain, and the other is a broad component consisting of a bile acid phospholipid-rich domain.

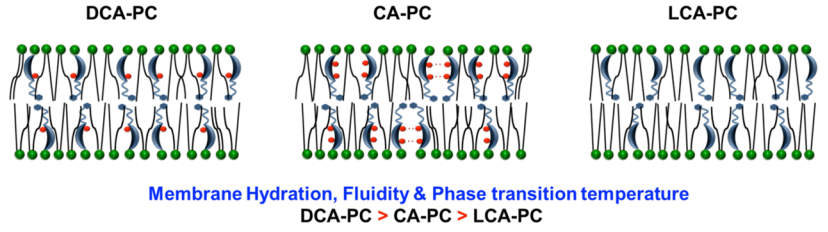

Figure 9. Schematic presentation of the effect of bile acid phospholipids on the membrane fluidity and phase transition of DPPC membranes.

complete separation of broad and sharp peak components where the T_m of the broad peak shifted to 30 °C and that of the sharp peak shifted to 39 °C (Figure 8). These results suggest that **LCA-PC** distorts packing in DPPC-poor domains to lower T_m, whereas it induces ordering of DPPC lipids in DPPC-rich domains, resulting in only 2 °C fall in T_m of DPPC-rich domains. Doping of 10 mol % of **DCA-PC** lowers peak height and induces the formation of two domains in which the T_m values of both broad and sharp peaks were not separated (Figure 8). Doping of more than 30 mol % of **DCA-PC** results in the separation of two-component domains. **DCA-PC** lowers main transition T_m to 28.7 °C with broadening on 70 mol % doping; as a result, the T_m of the sharp peak component is shifted to 23 °C (Figure 8). This suggests that **DCA-PC** integration perturbs acyl chain packing, resulting in lowering of T_m for both broad and sharp peak components. **CA-PC** showed distinct thermograms reducing peak height on 30 mol % doping followed by a rise in peak height at higher doping. Changes in T_m for broad and sharp components presented interesting behavior across **CA-PC** doping concentrations (Figure 8). **CA-PC** results in the formation of sharp and broad component peaks with no change in T_m on 10% doping, whereas 30 mol % doping results in the separation of broad and sharp T_m. Doping of 70 mol % of **CA-PC** did not change T_m between broad and sharp components. These results clearly suggest that **CA-PC** molecules may form self-assembled domains in DPPC membranes at higher doping concentrations (Figure 9). These self-aggregates do not allow phase separation/transition of DPPC-rich and DPPC-poor domains and do not allow further lowering of T_m. Therefore the presence of hydroxyl

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groups at C_7 and C_{12} positions essentially drive self-assemblies that cause sharpening of the main transition peak.

CONCLUSIONS

Lipid−lipid interactions, especially interactions of synthetic phospholipids with membranes, are critical for delivery of therapeutics, as phospholipids have been commercialized for efficient drug encapsulation and delivery. Therefore we divulged the interactions of three bile acid-based phospholipids with model membranes. Fluorescence and calorimetric studies revealed that the number of free hydroxyl groups on the concave side of bile acids influences their packing in DPPC membranes (Figure 9). The presence of one hydroxyl group in **DCA-PC** disrupts membrane packing and induces maximum fluidity with more hydration. Therefore doping of **DCA-PC** abolishes T_m of membranes. **LCA-PC** molecules induce gel phase and rigidify the membranes on doping due to the absence of free hydroxyl group. **LCA-PC** doping does not increase membrane hydration; however, it induces broadening of phase transition without lowering of T_m due to hydrophobic bile acid-acyl chain interactions. **CA-PC** due to the presence of two free hydroxyl groups induces membrane hydration and disrupts the membranes. Higher doping of **CA-PC** sharpens the phase transition and does not allow the abolishment of phase transition. **CA-PC** may induce self-association and allow the hydrophobic bile acid acyl chain interactions. Therefore these new interlipidic interactions between bile acid phospholipids and model membranes can influence drug encapsulation and drug release efficacies that will be explored in the future.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS:

DPH: 1,6-diphenyl-1,3,5-hexatriene; DPPC: dipalmitoylphosphatidylcholine; DMPC: dimyristoylphosphatidylcholine; DSC: differential scanning calorimetry; LCA: lithocholic acid; DCA: deoxycholic acid; CA: cholic acid

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