# Role of Cholesterol in Interaction of Ionic Liquid with Model Lipid Membranes and Associated Permeability

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**Abstract**: In this work, we have investigated the impact of composition of cholesterol in the lipid membrane composed of phosphatidylcholine (POPC) or phosphatidylglycerol (POPG) on the membrane permeability induced by 1-dodecyl-3-methylimidazolium bromide ($[C_{12}MIM]^+Br^-$) ionic liquid using various biophysical techniques. We investigated four different compositions of cholesterol (10, 20, 30, and 40 mole%) both with POPC and POPC phospholipids. Membrane permeability was determined using steady-fluorescence-based dye leakage assay. Further, interaction of ionic liquid with lipid membranes was investigated using ζ-potential measurements, and dynamic light scattering for measuring the size distribution. POPC and POPG membranes both show a reduction in $[C_{12}MIM]^+$ induced membrane permeability in the presence of cholesterol which continues with a further increase in cholesterol content. The overall reduction in membrane permeability is more in POPG LUVs in the presence of 30 and 40 mol% cholesterol content. Besides this, cholesterol also impacts the $[C_{12}MIM]^+Br^-$-induced fusion of POPC and POPG LUVs at higher ionic liquid concentrations. POPG membranes become more fusion prone in the presence of cholesterol as compared to POPC lipid membrane.

## 1. Introduction

Cholesterol plays an important role in the functioning of eukaryotic cells and is mainly located in the plasma membrane, where it varies up to an extent of 50 mol% of total lipid content.[1] It acts as a precursor molecule for the synthesis of steroidal hormones, bile salts, and vitamins.[2] Interaction of cholesterol with various lipid components enhances the mechanical strength of membranes,[3] regulates their fluidity,[4] makes the membranes less permeable to water or small molecules,[5] and also induces changes in the phase behavior of the membrane.[5c, 6] The ordering[7] and condensing effects[8] are the most common effects of cholesterol at the molecular level.

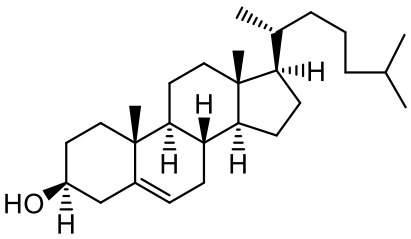


**Figure 1.** Molecular structure of cholesterol.

Cholesterol consists of a hydrophobic tetracyclic sterol ring covalently bonded to a hydrophilic hydroxyl group (**Figure 1**). On adding cholesterol to the lipid bilayers, phospholipid head-groups shield the hydrophobic sterol ring of cholesterol in the lipid bilayer. This prevents the exposure of the non-polar part of cholesterol to the aqueous medium and avoids unfavorable free-energy conformations. This molecular organization of cholesterol within a lipid bilayer induces condensing effect[9] on the lipid bilayer by reducing the surface area per lipid molecule or by increasing the order of lipid acyl chains. This results in the formation of cholesterol-rich domains commonly known as lipid-rafts which are known to play a crucial role in various cellular activities like signaling,[10] apoptosis,[11] membrane fusion,[12] and membrane trafficking.[13] Lipid rafts are responsible for reducing the area per lipid molecule which consequently leads to the increased thickness of lipid bilayer and decreased membrane permeability.[14] The morphology and functioning of the cellular system are quite complex to work with. This makes the understanding of the effect of cholesterol and its amount, which varies with the cell type, quite difficult.[15] Therefore, the biomimicking model membranes (liposomes) doped with cholesterol are often used to study the role of cholesterol in dictating the biophysical properties of lipid bilayers.[16] In literature, there are few reports in which the interaction of ionic liquid was studied with

cholesterol-containing model lipid membranes. Wiedmer *et al*. have studied the interaction of trioctylmethylphosphonium acetate ionic liquid with liposomes constituting PC with and without cholesterol using differential scanning calorimetry and nanoplasmonic sensing techniques.[17] Similar studies were also conducted by the same group using the same ionic liquid on LUVs and MLVs made of eggPC/eggPG with and without cholesterol using small-angle X-ray scattering.[18] They have found that loss of phospholipid content by ionic liquid is slower in cholesterol-containing LUVs as compared to pure vesicles.

In this work, impact of cholesterol on interaction of $[C_{12}MIM]^+Br^-$ ionic liquid with LUVs made with zwitterionic POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and anionic POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1´-rac-glycerol) (sodium salt)) phospholipids was studied. The LUVs made of POPC or POPG and cholesterol in molar ratios of 9:1, 8:2, 7:3, and 6:4 were tested for $[C_{12}MIM]^+$ induced permeability. Calcein-based dye leakage assays were performed to study the kinetics of membrane permeabilization from the LUVs. ζ-potential measurements were employed to determine the loading capacity/binding affinity of ionic liquid towards LUVs and DLS was used to study the size distribution of LUVs in the absence and presence of ionic liquid. The effect of higher concentrations of ionic liquid on cholesterol-containing POPC and POPG LUVs was also evaluated using DLS, FRET pair-based probe dilution assay, and ζ-potential measurements.

## 2. Experimental

### 2.1. *Chemicals*

The powdered form of Phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (> 99%), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG) (> 99%), Cholesterol (Ovine wool, > 98%), L-α-Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Ammonium Salt) (Rho-PE) (> 99%), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-PE) (> 99%) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Anhydrous sodium phosphate monobasic (AR grade) (99%), calcein extrapure (AR grade) and Triton X-100 (molecular biology grade) were purchased from Sisco Research Laboratories Pvt. Ltd. (Maharashtra, India). Sephadex G-50 was purchased from Sigma Aldrich, India. 1-Bromododecane (98%), 4-amino-3-hydroxy-1-naphthalene sulfonic acid (99%), and sodium dithionite

(DTN) (> 85%) were purchased from Alfa Aesar, India. Tris hydrochloride 99% (Molecular biology grade), sodium chloride 99.5% (AR grade), sulphuric acid 98% (AR grade) were purchased from Loba Chemie, Mumbai, India. Diethylether (AR grade), and sodium hydroxide pellets (AR grade) were purchased from SD fine-chem limited, (Mumbai, India). Ammonium heptamolybdate tetrahydrate, sodium sulphite anhydrous (>98%) were purchased from Merck, India. Sodium metabisulfite (98.5%) was purchased from Fisher Scientific, India. 1-Methyl imidazole (> 99%) was purchased from Spectrochem, India. Perchloric acid 70% (AR grade) was purchased from Qualikems Fine Chem Pvt. Ltd., India. Ionic liquid $[C_{12}MIM]^+Br^-$ was synthesized as per previous report[19] and its characterization and synthesis details were also shown.

### 2.2. *Methods*

#### 2.2.1. *Preparation of LUVs*

For the dye leakage, dynamic light scattering, ζ-potential, lipid mixing measurements POPC and POPG phospholipids-based LUVs containing variable amounts of cholesterol were prepared. For the preparation of LUVs, a 5 mM solution of POPC and POPG along with 10, 20, 30, and 40 mol% of cholesterol were dissolved in chloroform. A thin film was formed on the walls of the glass vial by removing chloroform under a gentle stream of nitrogen. To further remove any residual chloroform, the sample was dried overnight under a vacuum. The rest protocol is similar to our previous report.[19]

#### 2.2.2. *Membrane Permeability Assay*

Membrane permeability assay was performed on the POPC and POPG LUVs composing variable amounts of cholesterol (0, 10, 20, 30, 40 mol%) encapsulated with self-quenched calcein dye at 70 mM concentration. Stock solution of $[C_{12}MIM]^+Br^-$ (100 mM) was prepared in 7.7 mM Tris HCl buffer containing 100 mM NaCl (pH 7.4). An appropriate amount of dye-filled cholesterol-containing POPC and POPG LUVs was added to the buffer containing $[C_{12}MIM]^+Br^-$ achieving a final phospholipid concentration of 0.275 ± 0.015 mM in each case. The rest procedure for dye leakage measurement is similar as described earler.[19] The solutions were gently mixed and transferred to quartz cuvette to perform fluorescence measurements (dead time = 30 s) using PerkinElmer LS-55 Luminescence spectrometer. Calcein emission was measured at 520 nm with the excitation wavelength set at 485 nm using an excitation and emission slit width of 10 nm, each. The percentage of dye-leakage was calculated by using the following equation

$$\% \text{ Dye leakage} = \frac{I_t - I_{0,t}}{I_{max,t} - I_{0,t}} \times 100$$

$I_{0,t}$ and $I_t$ are the observed fluorescence intensity in the absence and presence of ionic liquid. $I_{max,t}$ is the florescence intensity obtained after addition of 1% Triton X-100. Separate set of controls $I_{max,t}$ and $I_{0,t}$ were recorded for each LUV preparation. The normalized data was smoothened by three-point averaging and plotted against time. All the measurements were repeated thrice and the reproducibility of results was obtained with standard deviation of less than 5%.

### 2.2.3. *Dynamic Light Scattering (DLS) and ζ-Potential Measurements*

A stock solution of 100 mM $[C_{12}MIM]^+Br^-$ ionic liquid was prepared in 7.7 mM Tris HCl buffer containing 100 mM of NaCl (pH 7.4). Size distribution measurements of cholesterol-containing POPC and POPG LUVs were performed in the absence and presence of variable concentrations (1-10 mM) of $[C_{12}MIM]^+Br^-$ ionic liquid at 25 °C using Malvern instrument (Zeta-sizer, Nano Series, nano-ZS, Malvern, U.K.). Samples were thermally equilibrated for 10 min before each measurement. The concentration of phospholipids in LUVs was fixed at 0.275 ± 0.015 mM in each measurement. ζ-potential measurements of cholesterol-containing POPC and POPG LUVs were measured in the absence and presence of variable concentrations (0.05, 0.2, 0.5, 0.75, 1 mM) of $[C_{12}MIM]^+Br^-$ at 25 °C. The ζ-potential measurements of cholesterol-containing POPC and POPG LUVs were also performed in the presence of a higher concentration of $[C_{12}MIM]^+Br^-$ (1-10 mM).

### 2.2.4. *Lipid Mixing Assay*

To measure lipid mixing during membrane fusion a probe dilution assay based on the mixing of LUVs containing FRET pairs was performed.[20] The POPC and POPG LUVs were composed of variable amounts (0, 10, 20, 30, 40 mol%) of cholesterol which also contains FRET pair probes (NBD-PE (donor) and Rho-PE (acceptor)) at a concentration of 1.5 mol% each. The rest protocol is similar as described earlier.[21] Fluorescence de-quenching of NBD-PE due to dilution of FRET probes into probe-free LUVs was monitored after 10 minutes of the addition of different concentrations of ionic liquid.

The percentage of lipid mixing was calculated using the equation

$$\% \text{ Lipid mixing} = \frac{I_t - I_{0,t}}{I_{max,t} - I_{0,t}} \times 100$$

where $I_t$ is the fluorescence emission intensity of NBD-PE at time t in the presence of ionic liquid, $I_{0,t}$ is the fluorescence intensity in the absence of ionic liquid. $I_{max,t}$ is the maximum fluorescence intensity obtained after addition of 1% (v/v) Triton X-100. A correction factor of 1.5 was applied to observed fluorescence in the last case as Triton X-100 is known to affect NBD-PE fluorescence.[21]

## 3. Results and Discussion

### 3.1. *Membrane Permeability Assay*

Owing to the surface-active behavior of imidazolium-based ionic liquids, they easily intercalate into the lipid membrane and show a detergent-like effect. This causes alteration in the membrane integrity and stability, which might lead to membrane permeabilization.[19, 21-22] Membrane permeability can be easily determined by measuring the amount of dye leaking out from the cholesterol-containing POPC and POPG LUVs as a function of $[C_{12}MIM]^+Br^-$ concentration and time. **Figures 2a-j** shows time-based leakage of calcein dye from the PC:Chol (10:0, 9:1, 8:2, 7:3, 6:4) and PG:Chol (10:0, 9:1, 8:2, 7:3, 6:4) LUVs as a function of variable $[C_{12}MIM]^+Br^-$ concentration. In our previous publication, we have studied the impact of $[C_{12}MIM]^+Br^-$ on membrane permeability of POPC and POPG LUVs. The POPC LUVs were found to be more leakage prone than POPG LUVs.[23] Similarly, PC:Chol LUVs were found to be leakier as compared to PG:Chol LUVs in the presence of $[C_{12}MIM]^+Br^-$. However, overall membrane permeability was found to be less than pure POPC and POPG LUVs. The resistance of POPC and POPG membranes against the ionic liquid increases with an increase in the amount of cholesterol. Similar kind of results was also observed in pure PC and PC/Chol LUVs in the presence of N-9 (nonionic), C31G (an amphoteric mixture of two surface-active molecules, C14 alkylamine oxide and C16 alkyl betaine (zwitterionic), BZK (cationic); and SDS (anionic) surfactants studied by Apel-Paz *et al*.[24] The time-based leakage kinetics of PC:Chol and PG:Chol LUVs after the addition of 1 mM $[C_{12}MIM]^+Br^-$ (**Figure 3**) was fitted with a sigmoidal equation ($\%\ dye\ leakage = a_0/(1 + e^{-(t-t_c)*k})$ which provides rate constant $k$, maximum dye leakage ($a_0$) and time ($t_c$) at which dye leakage reduced to $a_0/2$ are shown in **Table 1**. In PC:Chol LUVs, the rate of leakage decreases with an increase in cholesterol content but in POPG LUVs containing 10 mol% of cholesterol, the rate of leakage is faster than in pure POPG LUVs. With a further increase in cholesterol content to 20 mol% rate of leakage decreases and remains constant on further increase in cholesterol content in

POPG LUVs (**Table 1**). This reduction in the extent of dye leakage on the addition of cholesterol in POPC and POPG LUVs might be due to an increase in rigidity of the lipid bilayer[25] which makes the membrane less permeable to the ionic liquid.

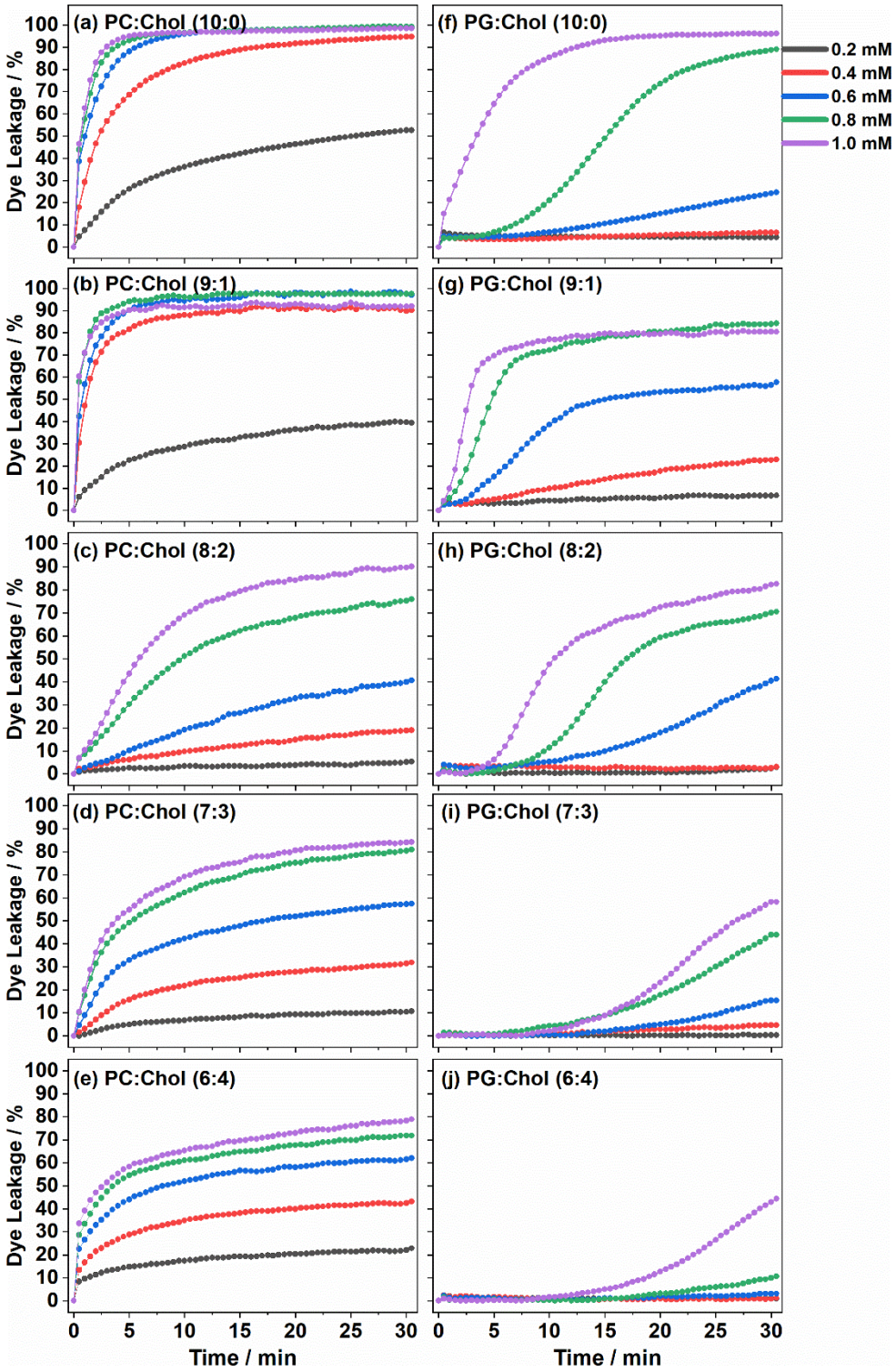


**Figure 2.** Time-based dye leakage in the presence of 0.2, 0.4, 0.6, 0.8, and 1 mM $[C_{12}MIM]^+Br^-$ from (a) PC:Chol (10:0), (b) PC:Chol (9:1), (c) PC:Chol (8:2), (d) PC:Chol (7:3), (e) PC:Chol (6:4), (f) PG:Chol (10:0), (g) PG:Chol (9:1), (h) PG:Chol

(8:2), (i) PG:Chol (7:3), and (j) PG:Chol (6:4) LUVs measured at 25 °C. These measurements were performed thrice and plots are the average of obtained data of three experiments. The expermental error for all these measurements is less than 3%.

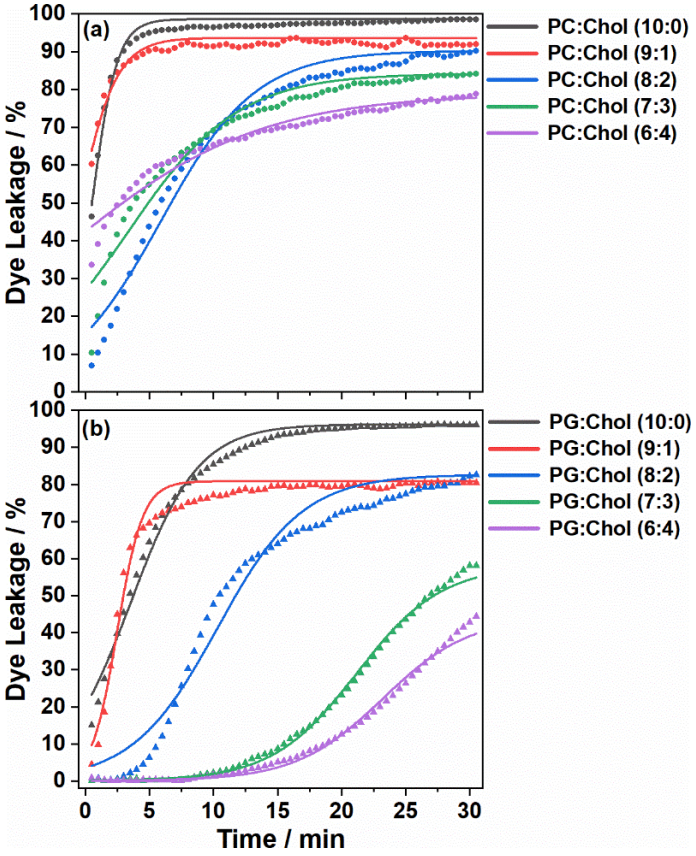


**Figure 3.** Time-based dye leakage from (a) PC:Chol (10:0, 9:1, 8:2, 7:3, 6:4), and (b) PG:Chol (10:0, 9:1, 8:2, 7:3, 6:4) LUVs upon addition of 1 mM $[C_{12}MIM]^{+}Br^{-}$. Solid lines represent sigmoidal fittings.

| System | k ($min^{-1}$) | $a_0$ (%) | $t_c$ (min) | Δζ (mV) |
|---|---|---|---|---|
| PC:Chol (10:0) | 1.01 ± 0.05 | 98.5 ± 0.43 | 0.50 ± 0.03 | 35.7 ± 3.0 |
| PC:Chol (9:1) | 0.67 ± 0.05 | 93.6 ± 0.12 | N.D. | 14.5 ± 1.0 |
| PC:Chol (8:2) | 0.27 ± 0.01 | 90.2 ± 0.55 | 5.9 ± 0.15 | 15.1 ± 0.2 |
| PC:Chol (7:3) | 0.23 ± 0.01 | 84.2 ± 0.68 | 3.3 ± 0.19 | 19.5 ± 3.7 |
| PC:Chol (6:4) | 0.14 ± 0.01 | 78.8 ± 0.57 | N.D. | 18.2 ± 2.4 |
| PG:Chol (10:0) | 0.38 ± 0.01 | 96.2 ± 0.34 | 3.53 ± 0.07 | 37.0 ± 2.4 |
| PG:Chol (9:1) | 1.00 ± 0.07 | 80.8 ± 0.33 | 2.5 ± 0.07 | 40.7 ± 0.2 |
| PG:Chol (8:2) | 0.30 ± 0.02 | 82.6 ± 0.75 | 10.3 ± 0.20 | 30.5 ± 2.2 |
| PG:Chol (7:3) | 0.30 ± 0.01 | 57.4 ± 0.52 | 21.50 ± 0.08 | 49.8 ± 4.5 |
| PG:Chol (6:4) | 0.29 ± 0.01 | 44.4 ± 0.33 | 23.2 ± 0.10 | 50.8 ± 1.7 |

**Table 1.** Parameters defining the leakage kinetics, and change in ζ-potential of PC:Chol and PG:Chol LUVs in the presence of $[C_{12}MIM]^+Br^-$ ionic liquid. N.D. refers to value that was too high or too low to yield meaningful fitting results.

### 3.2. *Binding Affinity of $[C_{12}MIM]^+Br^-$ towards Cholesterol-containing POPC and POPG LUVs*

To better understand the interaction of the ionic liquid with cholesterol-containing LUVs, we have determined the binding affinity/loading capacity of $[C_{12}MIM]^+Br^-$ towards PC:Chol and PG:Chol LUVs using ζ-potential studies. POPC LUVs in the absence of cholesterol have a slightly negative ζ-potential value while the cholesterol doped POPC LUVs show less negative (for 10 mol% cholesterol) and slightly positive (for 20, 30, and 40 mol% cholesterol) as shown in **Figure 4a**. On the other hand, cholesterol-containing POPG LUVs have higher negative ζ-potential values than the pure POPG LUVs, with an exception of POPG LUVs containing 20 mol% cholesterol which show slightly lower ζ-potential values as shown in **Figure 4b**. **Figures 4a** and **b** show the change in ζ-potential of cholesterol-containing POPC and POPG LUVs in the absence and presence of 1 mM $[C_{12}MIM]^+Br^-$. The addition of positively charged $[C_{12}MIM]^+$ cation into the LUVs increases the ζ-potential values towards less negative or positive values. The absolute change in ζ-potential values provides information about the loading capacity/binding affinity of ionic liquid towards LUVs. The overall change in ζ-potential values (Δζ) of PC:Chol LUVs in the presence of 1 mM $[C_{12}MIM]^+Br^-$ decreases with an increase in cholesterol content (**Table 1**). This means that the loading capacity/binding affinity of ionic liquid towards PC:Chol LUVs decreases with an increase in cholesterol content. The permeability trend of PC:Chol LUVs (10:0, 9:1, 8:2, 7:3, 6:4) is in agreement with the ability of $[C_{12}MIM]^+$ to bind with PC:Chol LUVs, both decrease with an increase in cholesterol content (**Table 1**). For POPG LUVs, we also took the absolute change in ζ-potential value as an indication of $[C_{12}MIM]^+$ loading on the membrane (**Table 1**). Seemingly, the loading capacity of PG:Chol (7:3 and 6:4) LUVs is more than other LUVs which contain a lesser amount of cholesterol content. This is in contrast to what is observed in the case of POPC LUVs which offer lower affinity to $[C_{12}MIM]^+$ cations when the cholesterol content is higher. Moreover, given the fact that POPG LUVs containing high cholesterol content are less prone to leakage, this observation once again underlines the fact that binding is not a primary factor that dictates membrane permeability.[21]

Cholesterol is also known to make the membrane more rigid by increasing the order of the lipid acyl chain by the formation of cholesterol rafts.[12] This effect also seems to contribute to the lower permeability of cholesterol-containing POPC and POPG LUVs. In our previous publication, we have shown that POPC LUVs are more leakage prone than POPG LUVs. A similar trend is also followed in PC:Chol and PG:Chol LUVs. The impact of cholesterol on LUVs permeability is more in the case of POPG LUVs than POPC LUVs. The overall decrease in membrane permeability on increasing cholesterol is much more in PG:Chol than in PC:Chol LUVs. The size of PC:Chol and PG:Chol LUVs remains intact in the presence of a 1 mM concentration of $[C_{12}MIM]^+Br^-$ (**Figures 5** and **6**).

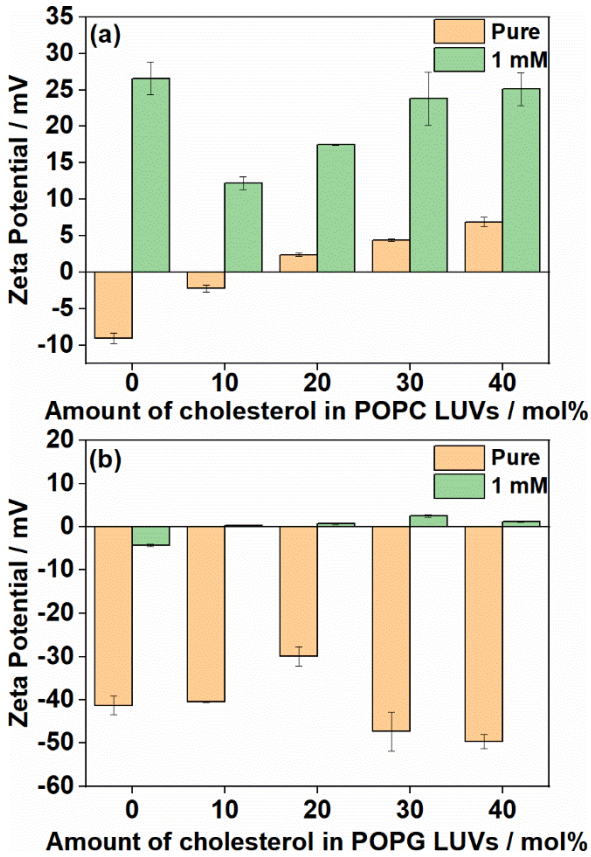


**Figure 4.** ζ-potential of (a) PC:Chol and (b) PG:Chol LUVs in the absence and presence of 1 mM $[C_{12}MIM]^+Br^-$ at 25 °C.

### 3.3. *Impact of Higher Concentration of $[C_{12}MIM]^+Br^-$ on the Size Distribution of Cholesterol-containing POPC and POPG LUVs*

Next, we have monitored the size distribution of cholesterol-containing LUVs as a function of a higher concentration of $[C_{12}MIM]^+Br^-$ using the DLS technique (**Figures 5** and **6**). The average size of all the studied LUVs is in the range of ~100-125 nm,

which remains unaffected in the presence of ionic liquid till 2 mM in PC:Chol (10:0), 4 mM in PC:Chol (9:1, 8:2), 5 mM in PC:Chol (7:3), 10 mM in PC:Chol (6:4), 3 mM in PG:Chol (10:0), 4 mM in PG:Chol (9:1, 8:2), and 5 mM in PG:Chol (7:3, 6:4) LUVs. Increasing the ionic liquid concentration beyond these values gives rise to new peaks positioned at ~1110 nm in pure POPC,[21] ~1350 nm in PC:Chol (9:1), ~1240 nm in PC:Chol (8:2), ~850 nm in PC:Chol (7:3), no visible effect of increased concentrations of ionic liquid on PC:Chol (6:4) LUVs was observed (**Figure 5**). In the cholesterol-containing POPG LUVs large size peak is positioned at ~955 nm in pure POPG,[21] ~955 nm in PG:Chol (9:1), ~830 nm in PG:Chol (8:2), ~750 nm in PG:Chol (7:3), and ~260 nm in PG:Chol (6:4) LUVs were observed (**Figure 6**). Similar kinds of results were also observed in DOPC/SM vesicles in the presence of $[C_2MIM]^+$ cations and due to the fusion of vesicles the size of LUVs increased from 100 nm to 1.7 μm.[26] Therefore, the larger size peaks observed in PC:Chol and PG:Chol LUVs correspond to the fused or aggregated vesicles. In our previous report, we have shown that this increase in the size of LUVs was due to the fusion of LUVs and not due to aggregation.

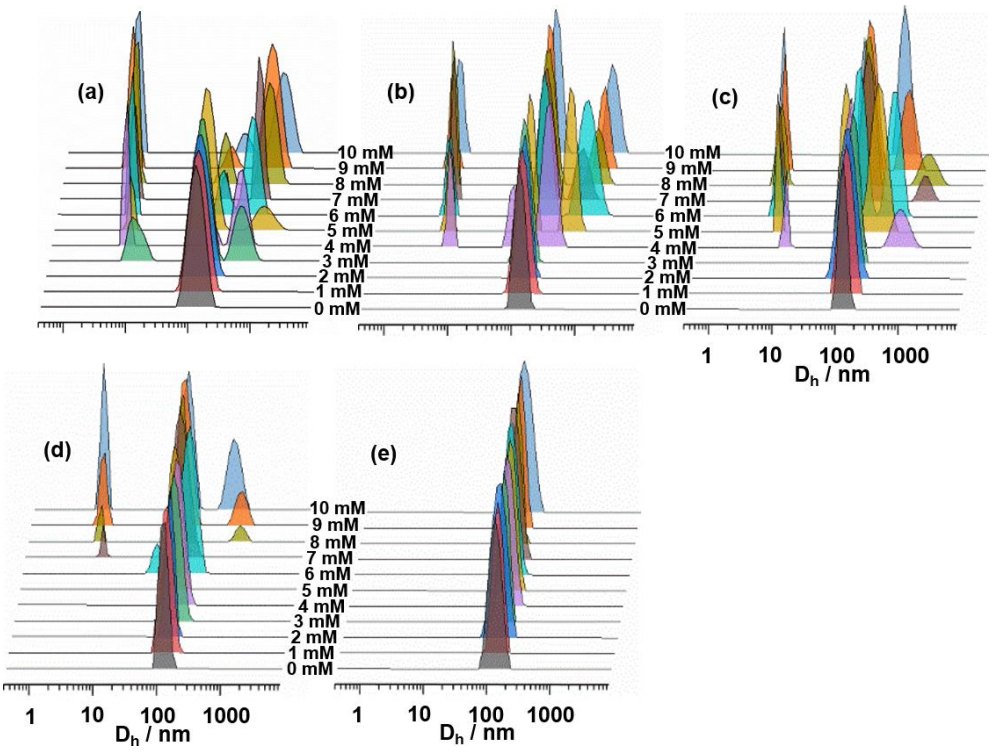


**Figure 5.** Hydrodynamic diameter ($D_h$) of (a) PC:Chol (10:0), (b) PC:Chol (9:1), (c) PC:Chol (8:2), (d) PC:Chol (7:3), and (e) PC:Chol (6:4) LUVs at 25 °C after the addition of $[C_{12}MIM]^+Br^-$ at the indicated concentrations. Total phospholipid concentration in the LUVs is 0.275 mM in all the cases.

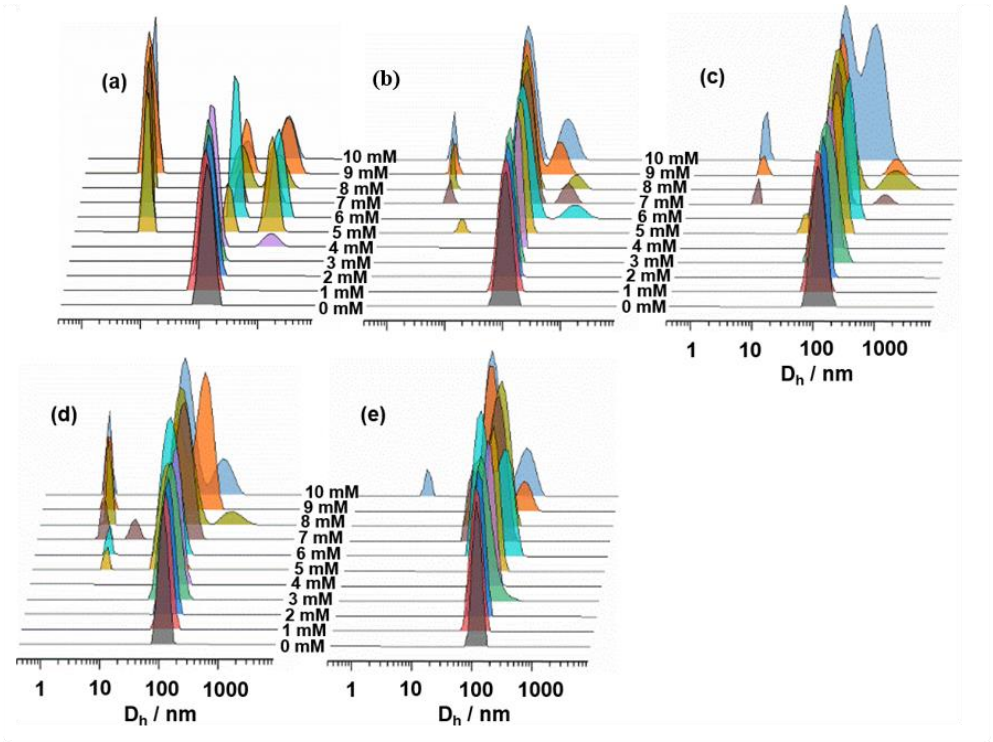


**Figure 6.** Hydrodynamic diameter ($D_h$) of (a) PG:Chol (10:0), (b) PG:Chol (9:1), (c) PG:Chol (8:2), (d) PG:Chol (7:3), and (e) PG:Chol (6:4) LUVs at 25 °C after the addition of $[C_{12}MIM]^+Br^-$ at the indicated concentrations. The total phospholipid concentration in the LUVs is 0.275 mM in all cases.

Besides peaks corresponding to large-sized particles, peaks at smaller size distributions in the range of ~5 to 50 nm were also observed in the DLS profiles of PC:Chol and PG:Chol LUVs, which correspond to micelles of $[C_{12}MIM]^+Br^-$ or mixed $[C_{12}MIM]^+$-lipid micelles. In the presence of ionic liquid, the formation of mixed micelles of ionic liquid and lipids has been already reported previously in the literature.[21, 27] The formation of mixed micelles might be due to the interaction of $[C_{12}MIM]^+Br^-$ micelles with liposomes which results in a population exchange among the micellar and lipidic phases. The results of DLS measurements confirm that the cholesterol-containing POPC and POPG LUVs show LUVs fusion in the presence of high ionic liquid concentrations. Only PC:Chol (6:4) LUVs do not show any increase in the size of LUVs in the studied ionic liquid concentration range which might be due to small-sized fused vesicles that are not detected by DLS.

### 3.4. *Lipid Mixing Assay*

To cross-check the possibility of formation of fused vesicles as indicated by DLS measurement, we have performed a fluorescence-based “probe dilution” assay to

measure the extent of lipid mixing among the merging LUVs as a function of increasing ionic liquid concentration.[28] In this assay, PC:Chol and PG:Chol LUVs containing 1.5 mol% of FRET pairs (NBD-PE and Rho-PE) were mixed with probe-free cholesterol-containing POPC and POPG LUVs in the molar ratio 1:4. Dilution of fluorescent probes leads to the de-quenching of NBD fluorescence which was monitored as a function of $[C_{12}MIM]^+Br^-$ concentration. The extent of lipid mixing in PC:Chol and PG:Chol LUVs in the presence of a variable concentration of ionic liquid after 10 minutes is shown in **Figures 7a** and **b**, respectively. The extent of lipid mixing confirms that the higher concentration of ionic liquid induces membrane fusion. The extent of lipid mixing at 5 mM ionic liquid concentration in PC:Chol LUVs follows the order: ~68% in PC:Chol (9:1) > ~63% in PC:Chol (8:2) > ~23% in PC:Chol (10:0)[21] > ~20% in PC:Chol (7:3) > ~12% in PC:Chol (6:4) LUVs (**Figure 7a**). While the PG:Chol LUVs (9:1, 8:2, 7:3, 6:4) show an almost similar percentage of lipid mixing that is ~76 % at 5 mM of $[C_{12}MIM]^+Br^-$ (**Figure 7b**) which is greater than pure POPG LUVs (~46% at 5 mM of $[C_{12}MIM]^+Br^-$[21]). When the amount of cholesterol is low (10 and 20 mol%) in POPC LUVs, the extent of lipid mixing is almost three times that of pure POPC LUVs. With a further increase in the concentration of cholesterol to 30 and 40 mol%, the extent of lipid mixing decreases rapidly. In PG:Chol LUVs (9:1, 8:2, 7:3, 6:4) the extent of lipid mixing is nearly the same at 5 mM ionic liquid concentration (**Figure 7b**). The overall extent of lipid mixing is higher in PG:Chol LUVs than in PC:Chol LUVs. For the fusion of two LUVs, their merging bilayers must overcome the hydration and electrostatic barriers to attain minimum spatial proximity.[29]

In our previous report, we have shown that fusion of POPC and POPG LUVs in the presence of a higher concentration of $[C_{12}MIM]^+$ is initiated by the short-range hydrophobic interactions among the merging bilayers but the extent of fusion is mainly dictated by long-range electrostatic interactions among the merging bilayers. The nearly neutral $[C_{12}MIM]^+$ bound POPG LUVs are more prone to fusion while the positively charged $[C_{12}MIM]^+$ bound POPC LUVs are less fusion prone. Similar was the trend of the fusion of POPC and POPG LUVs even after the addition of cholesterol at varying compositions in the LUVs which means in the presence of $[C_{12}MIM]^+$, PG:Chol LUVs are more fusion prone than PC:Chol LUVs (**Figures 7a, b** and **8**). The net surface charge carried by the PC:Chol LUVs at 5 mM ionic liquid concentration is 12.0 ± 3.12 mV in pure POPC,[21] 4.46 ± 0.70 mV in PC:Chol (9:1), 8.7 ± 1.54 mV in PC:Chol (8:2), 19.0 ± 3.73 mV in PC:Chol (7:3), 30.4 ± 4.38 mV in PC:Chol (6:4)

LUVs. From these values, it is clear that all the PC:Chol LUVs carry a net positive charge at 5 mM.

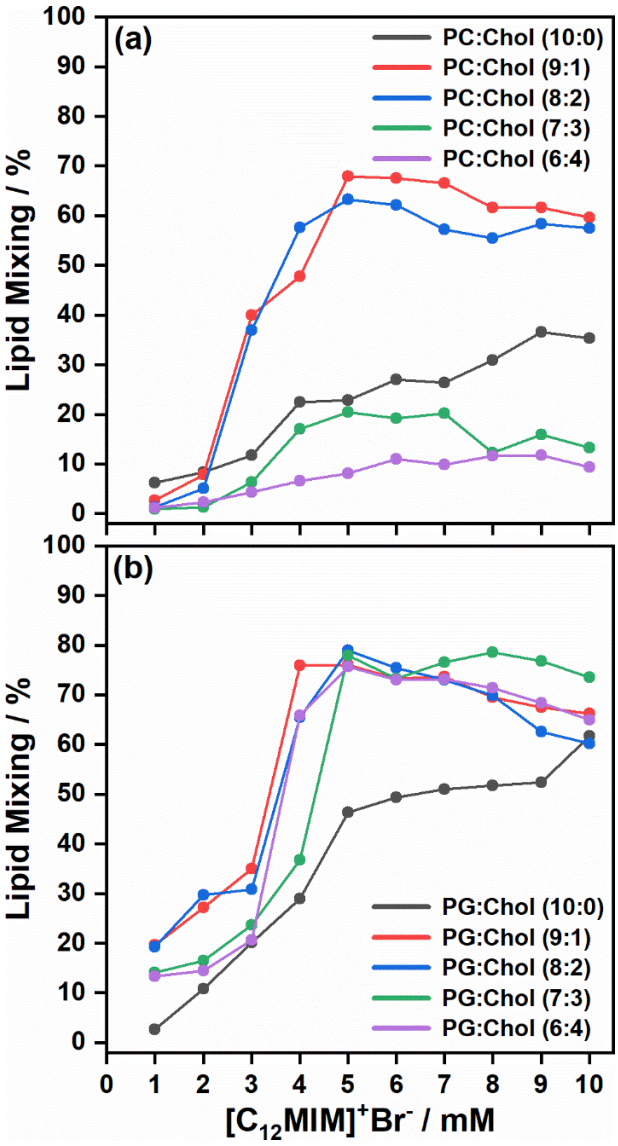


**Figure 7.** The extent of lipid mixing in (a) PC:Chol and (b) PG:Chol LUVs after 10 minutes of addition of a variable concentration of $[C_{12}MIM]^{+}Br^{-}$ at 25 °C.

the ionic liquid concentration which prevents their closest approach and lowers the degree of fusion. The greater the positive charge on the surface of LUVs in the presence of ionic liquid lesser will be the chances of LUVs fusion. While in PG:Chol LUVs at 5 mM ionic liquid concentration value of ζ-potential is 2.57 ± 0.74 mV in pure POPG,[21] 0.18 ± 0.06 mV in PG:Chol (9:1), 2.73 ± 0.2 mV in PG:Chol (8:2), 4.06 ± 1.2 mV in PG:Chol (7:3), and 8.94 ± 1.92 mV in PG:Chol (6:4) LUVs. On comparing the ζ-potential of PC:Chol LUVs with PG:Chol LUVs in the presence of 5 mM ionic liquid, we found that PG:Chol LUVs have nearly neutral or small positive charge on them and

show greater LUVs fusion which was observed in lipid mixing assay. The results of ζ-potential measurements of PC:Chol and PG:Chol LUVs in the presence of a variable concentration of $[C_{12}MIM]^+Br^-$ is shown in **Figure 8**.

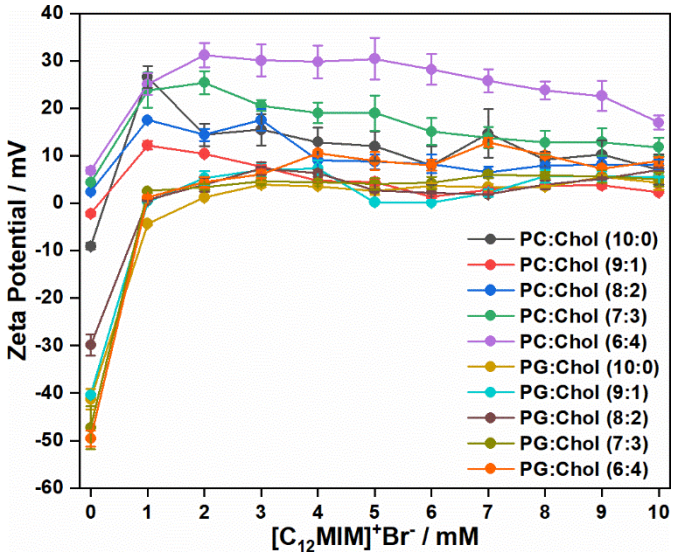


**Figure 8.** The change in ζ-potential of PC:Chol (10:0, 9:1, 8:2, 7:3, 6:4) and PG:Chol (10:0, 9:1, 8:2, 7:3, 6:4) LUVs as a function of $[C_{12}MIM]^+Br^-$ concentration.

## 4. Conclusions

In this work, we have evaluated the impact of variation of cholesterol content in the interaction of bio-mimicking membranes made of zwitterionic POPC and negatively charged POPG containing cholesterol in the molar ratios of 9:1, 8:2, 7:3, 6:4 with 1-dodecyl-3-methylimidazolium bromide ($[C_{12}MIM]^+Br^-$) ionic liquid. Both POPC and POPG LUVs show a reduction in $[C_{12}MIM]^+$ induced membrane permeability in the presence of cholesterol and this reduction in membrane permeability continues with a further increase in cholesterol content. The overall reduction in membrane permeability is more in POPG LUVs in the presence of 30 and 40 mol% cholesterol content. Besides this, cholesterol was also found to impact the $[C_{12}MIM]^+Br^-$-induced fusion of POPC and POPG LUVs at higher ionic liquid concentrations. PG:Chol LUVs were found to be more fusion prone than pure POPG and PC:Chol LUVs.

## 5. Credit Author Statement

The work reported in this chapter is under preparation for publishing. The contribution of each author to this work is as follows: **Sandeep Kumar**: Conceptualization, synthesis of ionic liquid, preparation of LUVs, performed dye leakage and lipid mixing assays, DLS and zeta potential studies. **Navleen Kaur**: DLS and zeta potential studies.

**Venus Singh Mithu**: Conceptualization, Funding acquisition, Project administration, Resources, Supervision.

**Notes**

**The author declare that they have no known competing financial interests**.

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## 7. References


[1] L. Finegold, M. A. Singer, CRC Press, Boca Raton, FL, **1993**, 137-157.

[2] P. A. Mayes, K. M. Botham, *Cholesterol, synthesis, transport and excretion*, a LANGE medical book, **2003**, 219.

[3] a) M. Y. El-Sayed, T. A. Guion, M. D. Fayer, *Biochemistry* **1986**, *25*, 4825-4832; b) M. Bloom, E. Evans, O. G. Mouritsen, *Q. Rev. Biophys.* **1991**, *24*, 293-397.

[4] R. A. Cooper, *J. Supramol. Struct.* **1978**, *8*, 413-430.

[5] a) A. Finkelstein, A. Cass, *Nature* **1967**, *216*, 717-718; b) W. K. Subczynski, A. Wisniewska, J. J. Yin, J. S. Hyde, A. Kusumi, *Biochemistry* **1994**, *33*, 7670-7681; c) J. H. Ipsen, G. Karlström, O. G. Mourtisen, H. Wennerström, M. J. Zuckermann, *Biochim. Biophys. Acta Biomembr.* **1987**, *905*, 162-172.

[6] M. R. Vist, J. H. Davis, *Biochemistry* **1990**, *29*, 451-464.

[7] a) E. Oldfield, M. Meadows, D. Rice, R. Jacobs, *Biochemistry* **1978**, *17*, 2727-2740; b) J. A. Urbina, S. Pekerar, H. B. Le, J. Patterson, B. Montez, E. Oldfield, *Biochim. Biophys. Acta Biomembr.* **1995**, *1238*, 163-176.

[8] D. Marsh, I. C. Smith, *Biochim. Biophys. Acta Biomembr.* **1973**, *298*, 133-144.

[9] a) E. Alipour, D. Halverson, S. McWhirter, G. C. Walker, *Annu. Rev. Phys. Chem.* **2017**, *68*, 261-283; b) B. N. DeWitt, R. C. Dunn, *Langmuir* **2015**, *31*, 995-1004. c) J. Huang, G. W. Feigenson, *Biophys. J.* **1999**, *76*, 2142-2157

[10] R. Sheng, Y. Chen, H. Y. Gee, E. Stec, H. R. Melowic, N. R. Blatner, M. P. Tun, Y. Kim, M. Källberg, T. K. Fujiwara, J. H. Hong, K. P. Kim, H. Lu, A. Kusumi, M. G. Lee, W. Cho, *Nat. Commun.* **2012**, *3*, 1-9.

[11] I. Tapas, *Cell Death Differ.* **2004**, *11*, S12-S16.

[12] S. T. Yang, A. J. B. Kreutzberger, J. Lee, V. Kiessling, L. K. Tamm, *Chem. Phys. Lipids* **2016**, *199*, 136-143.

[13] M. A. Alonso, J. Millán, *J. Cell Sci.* **2001**, *114*, 3957-3965.

[14] B. Bu, M. Crowe, J. Diao, B. Ji, D. Li, *Soft Matter* **2018**, *14*, 5277-5282.

[15] G. Van Meer, D. R. Voelker, G. W. Feigenson, *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 112-124.

[16] M. Eeman, M. Deleu, *Biotechnol. Agron. Soc. Environ. Biotechnol.* **2010**, *14*, 719-736.

[17] G. Russo, J. Witos, A. H. Rantamäki, S. K. Wiedmer, *Biochim. Biophys. Acta Biomembr.* **2017**, *1859*, 2361-2372.

[18] I. Kontro, K. Svedström, F. Duša, P. Ahvenainen, S. K. Ruokonen, J. Witos, S. K. Wiedmer, *Chem. Phys. Lipids* **2016**, *201*, 59-66.

[19] S. Kumar, H. A. Scheidt, N. Kaur, T. S. Kang, G. K. Gahlay, D. Huster, V. S. Mithu, *Langmuir* **2019**, *35*, 12215-12223.

[20] D. K. Struck, D. Hoekstra, R. E. Pagano, *Biochemistry* **1981**, *20*, 4093-4099.

[21] S. Kumar, N. Kaur, V. S. Mithu, *Phys. Chem. Chem. Phys.* **2020**, *22*, 25255-25263

[22] a) K. O. Evans, *Colloid Surf. A - Physicochem. Eng. Asp.* **2006**, *274*, 11-17; b) N. Kaur, M. Fischer, S. Kumar, G. K. Gahlay, H. A. Scheidt, V. S. Mithu, *J. Colloid Interface Sci.* **2021**, *581*, 954-963; N. Kaur, M. Fischer, S. Kumar, G. K. Gahlay, H. A. Scheidt, V.S. Mithu, *J. Magn. Reson. Open* **2022**, *10-11*, 100036.

[23] a) S. Kumar, H. A. Scheidt, N. Kaur, A. Kaur, T. S. Kang, D. Huster, V. S. Mithu, *J. Phys. Chem. B* **2018**, *122*, 6763-6770; b) S. Kumar, M. Fischer, N. Kaur, H. A. Scheidt, V.S. Mithu, *J. Phys. Chem. B* **2022**, *126*, 174-183; c) N. Kaur, S. Kumar, Shiksha, G. K. Gahlay, V. S. Mithu, *J. Phys. Chem. B* **2021**, *125*, 3613-3621.

[24] M. Apel-Paz, G. F. Doncel, T. K. Vanderlick, *Langmuir* **2005**, *21*, 9843-9849.

[25] T. M. Ferreira, F. Coreta-Gomes, O. S. Ollila, M. J. Moreno, W. L. Vaz, D. Topgaard, *Phys. Chem. Chem. Phys.* **2013**, *15*, 1976-1989.

[26] E. H. Hayakawa, E. Mochizuki, T. Tsuda, K. Akiyoshi, H. Matsuoka, S. Kuwabata, *PLoS One* **2013**, *8*, e85467.

[27] B. Jing, N. Lan, J. Qiu, Y. Zhu, *J. Phys. Chem. B* **2016**, *120*, 2781-2789.

[28] G. Cevc, H. Richardsen, *Adv. Drug Deliv. Rev.* **1999**, *38*, 207-232.

[29] a) S. W. Burgess, T. J. McIntosh, B. R. Lentz, *Biochemistry* **1992**, *31*, 2653-2661; b) S. Ohki, *Biochim. Biophys. Acta Biomembr.* **1982**, *689*, 1-11.