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Mixing of oxidized and bilayer phospholipids

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ABSTRACT

Composition and phase dependence of the mixing of 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), with the oxidized phospholipid, 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC) were investigated by characterizing the aggregation states of DPPC/PGPC and DOPC/PGPC using a fluorescence quenching assay, dynamic light scattering, and time-resolved fluorescence quenching in the temperature range 5–60 °C. PGPC forms 3.5 nm radii micelles of aggregation number 33. In the gel phase, DPPC and PGPC fuse to form mixed vesicles for PGPC molar fraction, $X_{PGPC} \leq 0.3$ and coexisting vesicles and micelles at higher X_{PGPC}. Data suggest that liquid phase DPPC at 50 °C forms mixed vesicles with segregated or hemi fused DPPC and PGPC for $X_{PGPC} \leq 0.3$. At 60 °C, DPPC and PGPC do not mix, but form coexisting vesicles and micelles. DOPC and PGPC do not mix in any proportion in the liquid phase. Two dissimilar aggregates of the sizes of vesicles and PGPC micelles were observed for all X_{PGPC} for $T \geq 22$ °C. DOPC–PGPC and DPPC–PGPC mixing is non-ideal for $X_{PGPC} > 0.3$ in both gel and fluid phases resulting in exclusion of PGPC from the bilayer. Formation of mixed vesicles is favored in the gel phase but not in the liquid phase for $X_{PGPC} \leq 0.3$. For $X_{PGPC} \leq 0.3$, aggregation states change progressively from mixed vesicles in the gel phase to component segregated mixed vesicles in the liquid phase close to the chain melting transition temperature to separated coexisting vesicles and micelles at higher temperatures.

1. Introduction

Oxidized phospholipids (OxPL) are implicated in various pathogeneses, because of which OxPL are considered cytotoxic [1–3] (Fig. 1). Membrane lipids upon oxidation do not just continue to be integrated within the membrane but transfer between cells [4]. Pathways involving specific receptor recognition as well as non-specific mechanisms of OxPL uptake by membranes and the genesis of OxPL toxicity are currently active areas of research. Non-specific trapping of OxPL at endocytotic sites rather than specific receptor mediation has been indicated to be the prevalent mechanism in some cell membranes [4]. The mixing behavior of OxPL with membrane lipids and the biophysical changes to the membrane bilayer are crucial to elucidating the mechanisms of OxPL induced toxicity. The increased susceptibility of apoptotic cells, whose membranes contain OxPL, to PLA_2 attack, appears to be directly linked to biophysical changes due to OxPL [5]. Possible link between biophysical changes in membranes induced by OxPL and associated pathologies motivate biophysical probing of OxPL included membranes. Evidence has accumulated for OxPL induced phenomena such as increase in membrane polarity and hydration, increase in lateral lipid mobility, membrane thinning, aggravated flip-flop, decrease in membrane order, and decrease in the bilayer chain melting temperature [6]. Contribution of the structural difference between bilayer lipids and OxPL to the bilayer properties, concentration and distribution of OxPL in the bilayer, and mixing behavior of bilayer lipids and OxPL are important questions in membrane biophysics. In this work mixing of the OxPL, PGPC, with DPPC and DOPC bilayers was investigated using a quenching assay of pyrene fluorescence, and Dynamic Light Scattering. Inclusion or exclusion of the OxPL in these model bilayers, where there are no endocytotic sites or receptors to facilitate trapping or uptake, is quite sensitive to the bilayer phase and temperature. It turns out that the uptake or integration of OxPL by bilayer lipids is rather limited. Association of bilayer lipids and OxPL is not preferred and in fact OxPL either segregate within a bilayer or are excluded from bilayers at temperatures above the chain melting transition (T_m). Unlike solubilization of bilayers by detergents, where bilayers are consumed by micelles at sufficient detergent concentration, the present mixture of PGPC and DPPC or DOPC behaves as a two-component system that exhibits mixing or demixing depending on mixture composition, temperature, and bilayer phase. A pattern with respect to temperature and bilayer phase, in the mixing behaviors of PGPC with bilayer forming phospholipids appears to emerge. Mixing is favored in the bilayer gel phase; but only up to a limited PGPC composition. PGPC and bilayer phospholipids are immiscible in the bilayer liquid phase at all compositions above T_m. However at temperatures within about 20 °C above T_m mixed vesicles with segregated PGPC and bilayer phospholipids or partially fused aggregates form followed by a separation into coexisting vesicles and mixed micelles of PGPC and bilayer phospholipids at higher

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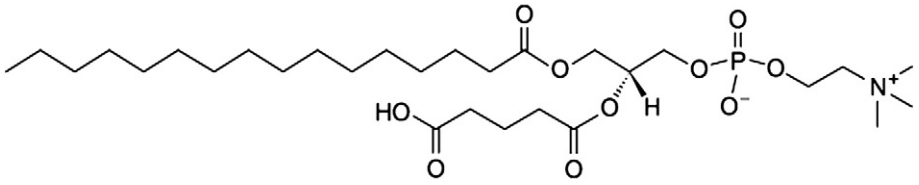

Fig. 1. Chemical structure of the oxidized phospholipid, PGPC.

temperatures. The thermodynamic phase of the bilayer is a key relevant property in the determination of the mixing behavior.

2. Materials and methods

2.1. Materials

The lipids, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and the oxidized lipid, 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC) were obtained from Avanti Polar lipids as lyophilized powders. Pyrene (optical grade, 99% Sigma) was the probe employed for fluorescence measurements and 3,4-dimethyl benzophenone (DMBP, 99% Sigma) was used as quencher. All samples were prepared in HEPES buffer (pH = 7.4).

2.2. Sample preparation

Small unilameller vesicles (SUV) were prepared by first dissolving calculated amounts for the required concentration in the stock volume of phospholipids (DPPC or DOPC) in ethanol. The ethanol solution was vortexed thoroughly to produce a clear solution which was then dried under dry N_2 flux to produce a film of lipid. Thereafter, the required amounts of the Hepes buffer (pH = 7.4) was added to the dry film to achieve the final concentration of 4 mM. The solutions were vortexed for 5 min which produces multi lamellar vesicles (MLV). The MLV solution was then sonicated for 20–25 min with Qsonica ultrasonicator processor (Model Q500) operating at 80% power amplitude to produce SUVs. The oxidized lipid, PGPC is freely soluble in water, forming micelles. The calculated amount of PGPC was dissolved in Hepes buffer directly to prepare the micellar solution. The final concentration of PGPC stock solution was 4 mM. Mixing of the individually prepared solutions of bilayer phospholipid SUV and PGPC were investigated under normal stirring conditions.

For the fluorescence quenching assay, two solutions were prepared: 4 mM DPPC or DOPC with 0.94 μM pyrene and 4 mM PGPC with 0.13 mM DMBP quenchers. Appropriate amounts of stock pyrene and DMBP solutions in ethanol were taken in two different glass vials. The ethanol solutions were then dried under dry N_2 flux to produce thin films. Lipid SUV and PGPC micellar stock solutions were then added to the vials with pyrene and DMBP thin films, respectively. These individual solutions were stirred overnight to ensure solubilization of pyrene in lipid SUV and DMBP in micelles.

For DLS measurements, the stock solutions of lipid SUV and PGPC micelles were diluted to 1 mM and were filtered through Whatman 100 nm pore size nylon syringe filter. Various amounts of the stock lipid SUV and PGPC micelles were then mixed to get different molar fractions of oxidized lipids.

Samples for time-resolved fluorescence quenching (TRFQ) measurements of PGPC micelle aggregation number were 4 mM PGPC solutions with 0.94 μM pyrene only and with 0.94 μM pyrene and 0.13 mM DMBP quenchers. Dry films of pyrene or pyrene and DMBP were made by evaporating the ethanol from the measured amounts, as appropriate for the final concentrations, of stock solutions of pyrene or pyrene and DMBP in ethanol. Required amount of PGPC solution was added to the dry film. The solution was stirred for about 12 h to solubilize the pyrene and DMBP into the micelles.

2.3. Methods

2.3.1. Fluorescence quenching assay

Mixing of DPPC or DOPC with PGPC was investigated by monitoring changes in the fluorescence spectral emission of pyrene solubilized in DPPC or DOPC bilayers upon addition of PGPC micelles containing DMBP quenchers. Steady state fluorescence emission spectra of pyrene were measured with a Fluoromax-4 Spectrometer (Horiba Scientific). The excitation wavelength was 335 nm and the emission spectra were recorded from 350 nm to 500 nm. Fluorescence emission spectrum of pyrene in bilayer phospholipid (DPPC or DOPC) solution was first recorded. PGPC micellar solution with DMBP quencher, as required for final PGPC molar composition, X_{PGPC}, values between 0 and 1 was added to this solution. Spectra were recorded at various time intervals following addition. A decrease in fluorescence emission intensities is a signature of quenching.

In this assay, two properties extracted from the fluorescence spectra, namely the I_1/I_3 ratio of the fluorescence intensities of the first (372 nm) to the third (383 nm) vibronic band of pyrene fluorescence and the fluorescence intensity of the 393 nm line were used to characterize the mixing behavior of PGPC and the bilayer phospholipids, DPPC and DOPC. The I_1/I_3 ratio of pyrene fluorescence is a measure of the polarity of the pyrene neighborhood. Typical value of I_1/I_3 is about 1 in vesicles of bilayer forming lipids and about 1.3 in micelles [7,8]. A value of 1.24 was measured for PGPC micelles in this work. Occurrence of quenching alone does not uniquely determine presence of mixing because probe/quencher encounters and transfers during collisions between vesicles and micelles without actual mixing can also result in quenching. However, as shown in the Results section, interpretation of quenching together with polarity and aggregate size measurements obtained from DLS leads to a better definition of the nature of mixing. Fluorescence quenching assays of bilayer/micelle mixing were performed at various temperatures in the range 5 to 60 °C. Sample temperature was maintained by a thermostat and circulating water bath.

2.3.2. Dynamic light scattering measurements (DLS)

DLS measurements were conducted to determine the hydrodynamic radii of the aggregates in DPPC/PGPC and DOPC/PGPC solutions using a DynaPro Nanostar Model WDPN06 (Wyatt Technologies), equipped with a GaAs laser (120 mW) operating at a nominal wavelength of 658 nm. The scattered light was collected at 90° by a solid state Single Photon Counting Module (SPCM) detector. The sampling time was set to an optimum value to obtain a fully decaying intensity correlation function (ICF), which was typically 10 s. The ICF's were single exponential decays with baselines that were unity within the precision of the measurements. The exponential fit to ICF yielded the translational diffusion coefficient (D_t) of the particles in the sample. The hydrodynamic radius (R_h) of the sample was then derived from D_t using the Stokes–Einstein equation [9]. The temperature of the sample solutions was controlled by an internal Peltier effect heat pump with an accuracy of ±0.01 °C. Samples were prepared at 23 °C by adding PGPC micelles to DPPC or DOPC vesicles and mixed by stirring and equilibrated at 22 °C in

the DLS equipment. Samples were then heated to the measurements temperatures 37, 50 and 60 °C.

2.3.3. Time resolved fluorescence quenching

In the TRFQ method, fluorescence probes and quenchers, being themselves hydrophobic, are dispersed in micelles and the quenched time-decay of the probe fluorescence is measured. The fluorescence decay curves of pyrene were obtained by time-correlated single photon counting using an FL900 lifetime measurement spectrometer of Edinburgh Analytical Instruments (EAI) with nanosecond flash lamp excitation. The decay curves, corrected for instrument response, were fitted to the Infelta–Tachiya model [10–12] using the Level 2 analysis software of EAI. The fit returns the number of quenchers per micelle. The concentration of quenchers being a known sample parameter, the concentration of micelles and hence the aggregation number is calculated. Details of the TRFQ method and the micellar quenching model are available in previous publications [13,14].

3. Results

3.1. PGPC forms micelles

Dynamic Light Scattering experiments on aqueous PGPC solutions showed the presence of micelles of hydrodynamic radii 3 to 4 nm (data included in Fig. 2a with data of DPPC + PGPC mixtures). This is about the size of the length of a PGPC molecule. Time resolved fluorescence decay curves of pyrene in aqueous PGPC solutions in the absence and presence of quenchers (DMBP) were measured at 37 °C. The quenched decay curve was fit to the Infelta–Tachiya model of fluorescence quenching in micelles [13,14]. The aggregation number derived from the fit was 33 ± 5. In DLS measurements of mixtures, aggregates of radii 3 to 4 nm, if indicated, were taken to be purely PGPC micelles.

3.2. Mixing of DPPC and PGPC

3.2.1. Dynamic light scattering ($0 \le X_{PGPC} \le 1$)

Hydrodynamic radii of aggregates in solutions of mixtures of DPPC and PGPC at various compositions, displayed in Fig. 2a, show the changes in aggregate sizes and types (whether vesicles or micelles) with PGPC molar fraction at the DPPC bilayer gel phase temperature of 22 °C. Experiments were conducted for several X_{PGPC} from 0 to 1. Vesicles were the only type of aggregates for $0 \le X_{PGPC} \le 0.3$. The starting DPPC SUV for the various compositions were independently prepared samples. The average and standard deviation in the peak position of the vesicle radii distribution of DPPC vesicles from nine measurements on independently prepared samples was 44 ± 16 nm. The change in size upon formation of mixed vesicles was within this standard deviation. For $X_{PGPC} = 0.2$ the radius was 48 ± 8 nm. Data for $X_{PGPC} = 0$, 0.2, 0.8, and 1 in Fig. 2a are representative examples. These results are indicative of complete mixing of DPPC and PGPC to form mixed vesicles for $X_{PGPC} \le 0.3$. At higher PGPC mole fractions, $X_{PGPC} > 0.3$, vesicles as well as smaller aggregates of the

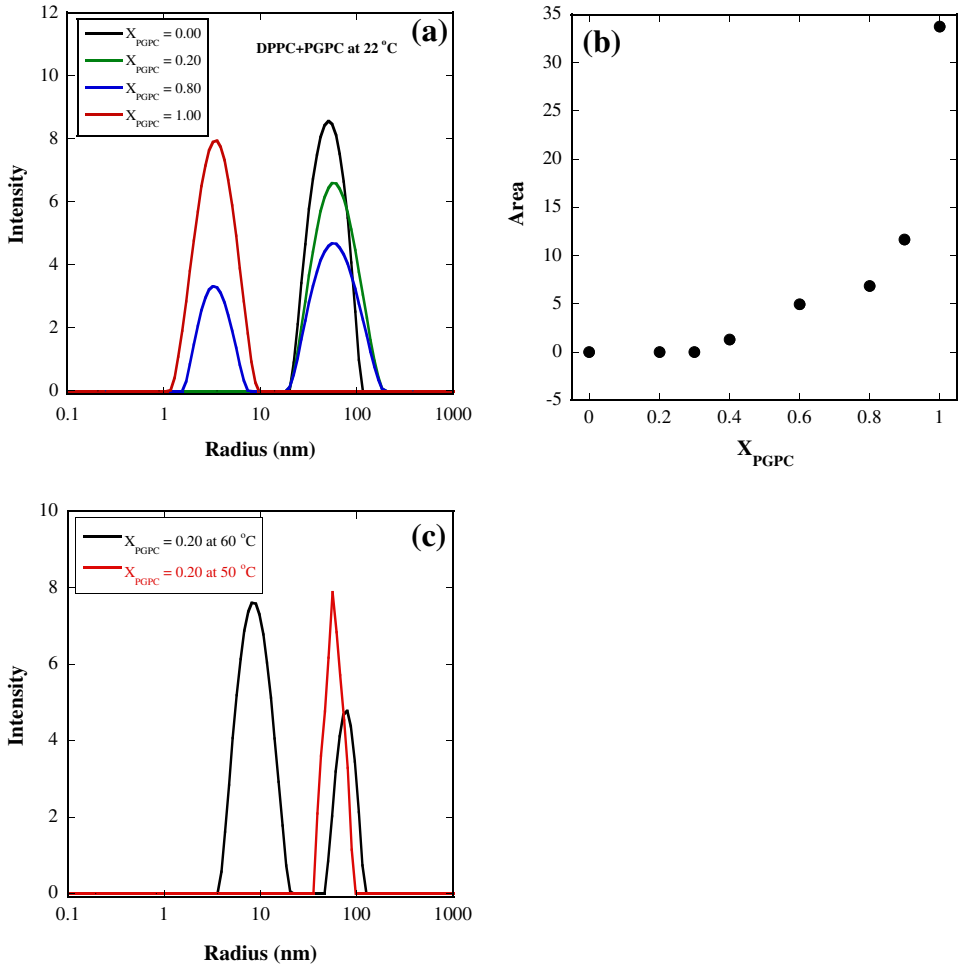

Fig. 2. Dynamic Light Scattering profiles for the hydrodynamic radii of DPPC + PGPC aggregates in the bilayer gel phase at T = 22 °C. [DPPC] + [PGPC] = 1 mM. (a) Size distribution indicates: only micelles (≈3.5 nm) for PGPC ($X_{PGPC} = 1$); mixed vesicles (≈50 nm) for $X_{PGPC} = 0.2$; micelles and vesicles for $X_{PGPC} = 0.8$; and only vesicles for DPPC ($X_{PGPC} = 0$). (b) Composition dependence of the area under the 3.5 nm peak. (c) Comparison of aggregate sizes for $X_{PGPC} = 0.2$ shows presence of only vesicles at T = 50 °C and coexisting micelles and vesicles at T = 60 °C.

size of PGPC micelles formed. No changes in the size distributions were observed over 5 h in all cases. The variation in the area under the 3.5 nm peak representing micelles with respect to the mixture composition is examined in Fig. 2b. The area begins to increase at X_{PGPC} between 0.3 and 0.4 indicating the transition from mixing to demixing. Aggregate size distributions were similar at 37 °C as well as 50 °C; namely presence of only vesicles for $X_{PGPC} \leq 0.3$ and coexisting vesicles and micelles for $X_{PGPC} > 0.3$. Although T = 50 °C is above the DPPC bilayer gel-liquid transition temperature of 41 °C, no observable change in the DLS derived aggregation states, over that in the gel phase, could be detected. However changes were observable at 60 °C.

The aggregation states at 50 and 60 °C are compared in Fig. 2c to show the temperature/bilayer phase induced change. DLS measurements at 60 °C on DPPC/PGPC mixtures for $X_{PGPC} = 0.2$ showed separation of the 22 °C vesicular aggregate into vesicles and aggregates (9 nm) larger than purely PGPC micelles. Results at 22 °C were reproduced upon cooling down from 60 °C. The 9 nm aggregates at 60 °C are perhaps PGPC rich mixed aggregates of DPPC + PGPC. It is a reasonable suggestion, albeit indirect, that smaller aggregates when twice as large as PGPC micelles contain some bilayer lipids. Mixed large cylindrical or disc like micelles of PGPC and DPPC are suggested. DLS overestimates the size when the scattering particles are not spherical [15]. On the contrary for $X_{PGPC} = 0.8$, the observed coexistence of vesicles and micelles (radius 3 to 4 nm) at 22 °C (shown in Fig. 2a) remained as such at 60 °C. Apparently at 60 °C where the DPPC bilayer is in the liquid phase, DPPC and PGPC separate into vesicles and PGPC micelles for $X_{PGPC} = 0.8$ just as in the gel phase. The data for $X_{PGPC} = 0.8$ at 60 °C are quite similar to the data obtained at 22 °C presented in Fig. 2a and hence not shown again.

3.2.2. Fluorescence quenching assays of DPPC/PGPC mixing ($X_{PGPC} = 0.2$ and 0.8)

The composition, temperature, and time dependence of the spectral properties of I_1/I_3 and the fluorescence intensity at 393 nm, following addition of PGPC micelles containing quencher to solutions of DPPC vesicles containing pyrene are summarized in Table 1. The spectra and details and implications of their characteristics are elaborated on below.

Spectra are shown in Fig. 3a for $X_{PGPC} = 0.2$ at 22 °C. Fluorescence intensities decreased to 69% of its initial value immediately upon addition of PGPC + DMBP micelles to DPPC vesicles containing pyrene at 22 °C for $X_{PGPC} = 0.2$. Furthermore the I_1/I_3 ratio of the fluorescence intensities increased from its value of about 1.04 in the DPPC bilayer to 1.22 upon addition of PGPC, signaling an increase in polarity of the pyrene neighborhood. A likely reason is the possibility of water penetration due to irregularity in the lipid packing caused by the introduction of the oxidized lipid whose architecture is different from that of the bilayer lipid [16]. The observed characteristics did not change significantly over a period of 4 h. The DLS data showing presence of only vesicles together with the fluorescence quenching and increase in polarity confirm that PGPC fuses with DPPC to form mixed vesicles at 22 °C when the molar fraction of PGPC in the solution mixture is 0.2.

Table 1
Pyrene fluorescence emission intensity at 393 nm and polarity of pyrene neighborhood, as determined by the ratio I_1/I_3, at various times following addition of PGPC micelles containing DMBP quenchers to DPPC bilayers containing pyrene. The emission intensity before addition is set to 1 with subsequent intensities expressed as fractions thereof.

Time (min)	$X_{PGPC} = 0.20$						$X_{PGPC} = 0.80$	
	T = 22 °C		T = 50 °C		T = 60 °C		T = 22 °C	
	I_{393}	I_1/I_3	I_{393}	I_1/I_3	I_{393}	I_1/I_3	I_{393}	I_1/I_3
Before	1.00	1.04	1.00	0.93	1.00	0.97	1.00	1.04
1	0.69	1.20	0.60	0.95	0.96	0.97	0.75	1.19
30	0.68	1.22	0.57	0.94	0.93	0.95	0.72	1.20
60	0.65	1.20	0.53	0.94	0.83	0.96	0.69	1.20
120	0.65	1.22			0.70	0.94	0.66	1.20
180	0.64	1.20			0.66	0.94	0.62	1.20
240	0.64	1.20			0.65	0.94	0.61	1.20

At 50 °C, while quenching was observed immediately upon PGPC addition to DPPC (Fig. 3b), an increase in I_1/I_3 from its value of 0.94 was not found for $X_{PGPC} = 0.2$ although DLS showed the only aggregation state to be vesicles. A possible explanation is that mixed vesicles do form, with however a lateral separation of DPPC rich and PGPC rich sections. Another possibility is the formation of hemi fused PGPC micelles on DPPC vesicles. DLS cannot distinguish between such hemi fused structures and vesicles. Pyrene remains sequestered in the more hydrophobic DPPC section accounting for the lower vesicle like value of I_1/I_3. Confocal microscopy images do show component segregation between fluorescently labeled OxPL and bilayer forming PL [5]. Lateral separation has been reported in POPC and OxPL mixed vesicles as well [6,16].

The fluorescence quenching assay data of DPPC/PGPC mixing at 60 °C for $X_{PGPC} = 0.2$ is shown in Fig. 3c. Adding PGPC + DMBP to DPPC + pyrene in the bilayer liquid phase at 60 °C to again the same final PGPC mole fraction of 0.2 as at 22 °C, did not produce any change toward increased polarity. The I_1/I_3 ratio of pyrene fluorescence remained at about 0.95 over a period of 4 h. The fluorescence intensity decreased to 83% of its initial value in the first hour and to 65% at the end of 4 h. This is in contrast to the immediate quenching accompanied by change in polarity in the gel phase. The slow development of quenching could be the result of slow exchange of pyrene and DMBP between aggregates. The exchange takes place via the aqueous medium and is known to be a very slow process [17–20]. Furthermore limited solubilization of phospholipids in PGPC micelles must also take place which results in the large 9 nm micelles observed by DLS at 60 °C. The lack of change in polarity of pyrene neighborhood particularly, and the slower development of quenching compared to that in the gel phase indicate preference for DPPC–PGPC separation and limited mixing. The fluorescence quenching assay results displayed in Fig. 3c support the DLS observation at 60 °C (Fig. 2c) that DPPC and PGPC do not mix completely in the bilayer liquid phase. DLS experiments were able to show that the mixing observed in the gel-phase lipids is reversed by raising the temperature. But fluorescence experiments cannot be conducted to show demixing of mixed vesicles because once mixed, pyrene and DMBP will not compartmentalize back. Notably however, the fluorescence assay results at the higher temperatures support the DLS results showing that heating the sample from the lower to higher temperature reproduces the results of mixing directly at the higher temperature.

Fluorescence quenching assay was also conducted on DPPC/PGPC for $X_{PGPC} = 0.8$ at 22 °C. DLS showed the presence of vesicles and micelles (Fig. 2a). The measured spectra are given in Fig. 4. The data presented so far (Figs. 2a and 3a) show that DPPC and PGPC do mix to about $X_{PGPC} \approx 0.3$. Addition of PGPC + quencher to DPPC + pyrene at 22 °C to a solution mole fraction of $X_{PGPC} = 0.8$ should show the effects of this level of mixing. The signatures of mixing were indeed observed as increase in the I_1/I_3 ratio from 1.04 to 1.20 and decrease in fluorescence intensity to 75% of its initial value before addition of quenchers.

3.3. Mixing of DOPC and PGPC. Character of the phase and T_m dependence of DOPC/PGPC mixing are quite similar to that of DPPC/PGPC

3.3.1. Dynamic light scattering ($0 \leq X_{PGPC} \leq 1$)

No sign of mixed vesicle formation could be found from the hydrodynamic radii distribution at any mixture composition (Fig. 5a) for T ≥ 22 °C. DOPC and PGPC coexist separately as DOPC vesicles and PGPC micelles at 22, 37, 50, and 60 °C. The radius of DOPC vesicles from seven independent measurements was 57 ± 12 nm. Data shown in Fig. 5a are for T = 37 °C and for representative X_{PGPC} of 0, 0.2, 0.8, and 1. Subjecting the DOPC/PGPC mixture to sonication did not produce mixing and the aggregates remained separated as vesicles and micelles. At T = 5 °C, Fig. 5b, DOPC and PGPC for $X_{PGPC} = 0.2$ aggregated as vesicles of size 50 nm and as smaller aggregates of radii about 9 nm just as in the case of DPPC/PGPC at 60 °C. Liquid phase DPPC/PGPC and DOPC/PGPC thus exhibit similarity at equivalent temperatures of about

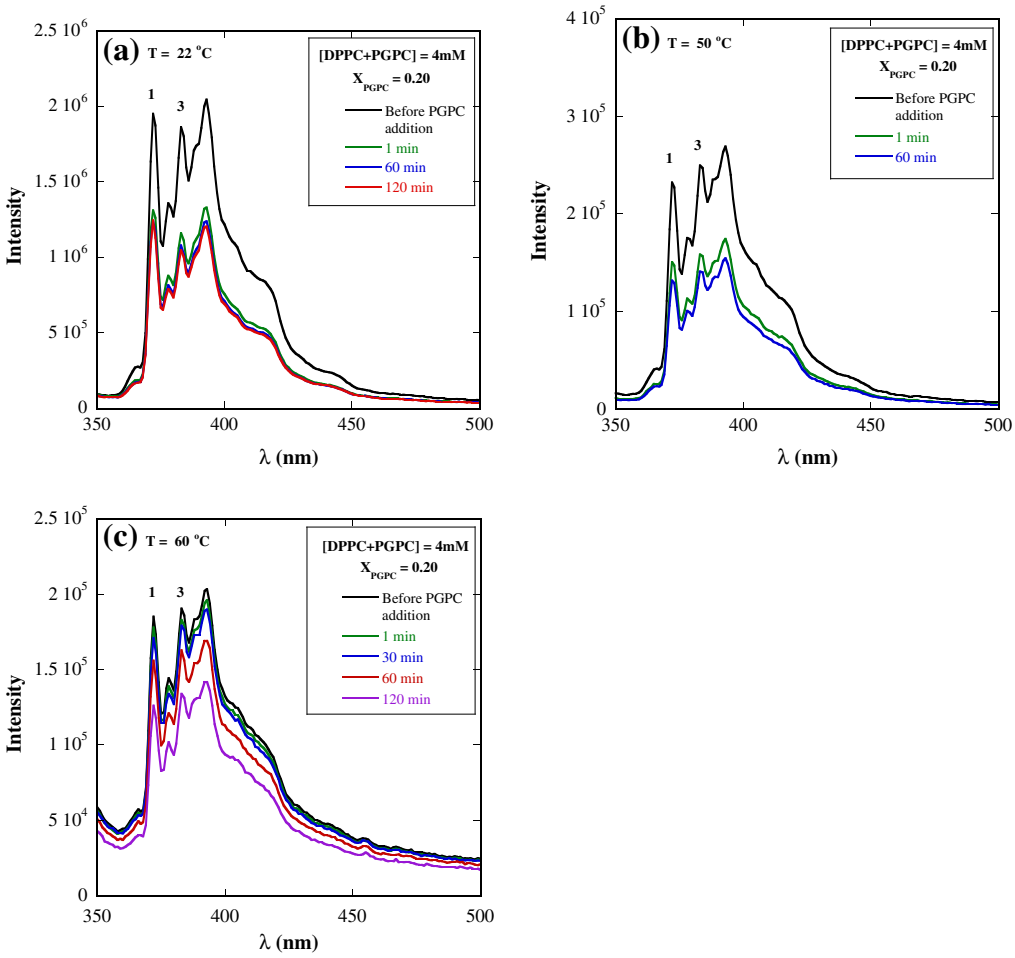

Fig. 3. Fluorescence spectra of pyrene in DPPC bilayers before and at the indicated times following addition of PGPC micelles containing DMBP quenchers. The final molar fraction of PGPC was 0.20. [DPPC] + [PGPC] = 4 mM. (a) T = 22 °C; (b) T = 50 °C; (c) T = 60 °C. Presence of quenching at T = 22 and 50 °C indicates mixing. No quenching in the first 1/2 h indicates absence of mixing.

20 °C above their melting points respectively of 41 °C and −17 °C, in the formation of coexisting vesicles and the second 9 nm DPPC or DOPC mixed PGPC cylindrical/disc like micelles.

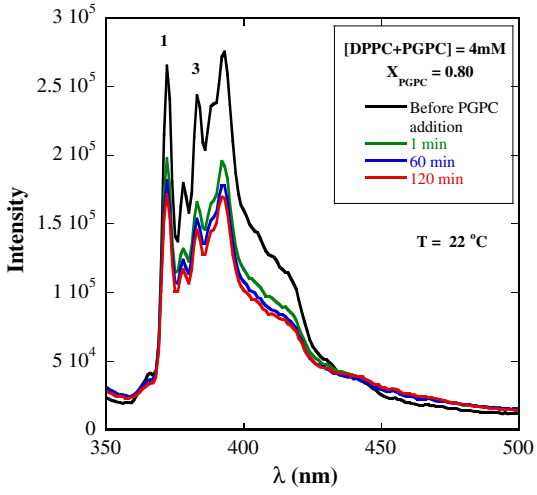

Fig. 4. Fluorescence spectra of pyrene in DPPC bilayers before and after adding PGPC to a final molar fraction of 0.80 PGPC at time intervals, shown in the legend, following addition of PGPC micelles containing DMBP quenchers. T = 22 °C. [DPPC] + [PGPC] = 4 mM. Presence of quenching is to be expected, consistent with the mixing known to be present to about $X_{PGPC} = 0.3$ (Fig. 2a and 3a).

3.3.2. Fluorescence quenching assays of DOPC/PGPC mixing ($X_{PGPC} = 0.2$)

Mixing behavior was investigated at 5, 22, 37, 50 and 60 °C. Fig. 6 is illustrative of the effect on the bilayer solubilized pyrene fluorescence, of adding PGPC + DMBP to DOPC + pyrene at 37 °C to a final solution concentration of $X_{PGPC} = 0.2$. No quenching was observed in the first 1/2 h. The fluorescence decreased by 5 to 10% after one hour. The I_1/I_3 ratio however remained at about 0.94 throughout. Results were similar at 22, 50 and 60 °C. The constancy of the polarity of pyrene neighborhood and the absence of immediate quenching and the slower development of quenching as compared to that in DPPC + PGPC are indicative of absence of DOPC/PGPC mixing at these temperatures. The combined DLS and fluorescence quenching assay data support the conclusion that DOPC and PGPC do not mix in any molar ratio when T ≥ 22 °C.

The similarity in aggregation states shown by DPPC/PGPC at 60 °C and DOPC/PGPC at 5 °C in the DLS experiments was further explored by the fluorescence quenching assay of DOPC/PGPC at 5 °C for $X_{PGPC} = 0.2$. Spectra are shown in Fig. 7. The quenching and polarity behaviors upon adding PGPC to DOPC were very similar to that in DPPC/PGPC at 60 °C (Fig. 3c). The I_1/I_3 ratio of pyrene fluorescence remained at about 1 over a period of 2 h. The fluorescence intensity decreased to 81% of its initial value at the end of 1 h. This is different from DOPC/PGPC mixing behavior for T ≥ 22 °C where there is no quenching in the first hour after addition of PGPC. The absence of quenching, lack of change in polarity and presence of vesicles and small micelles (3 to 4 nm radii) were taken to be evidence of separation of DOPC and PGPC for T ≥ 22 °C. However the remarkable similarity in the coexistence of vesicles and the smaller 9 nm aggregates and the slow development of quenching without

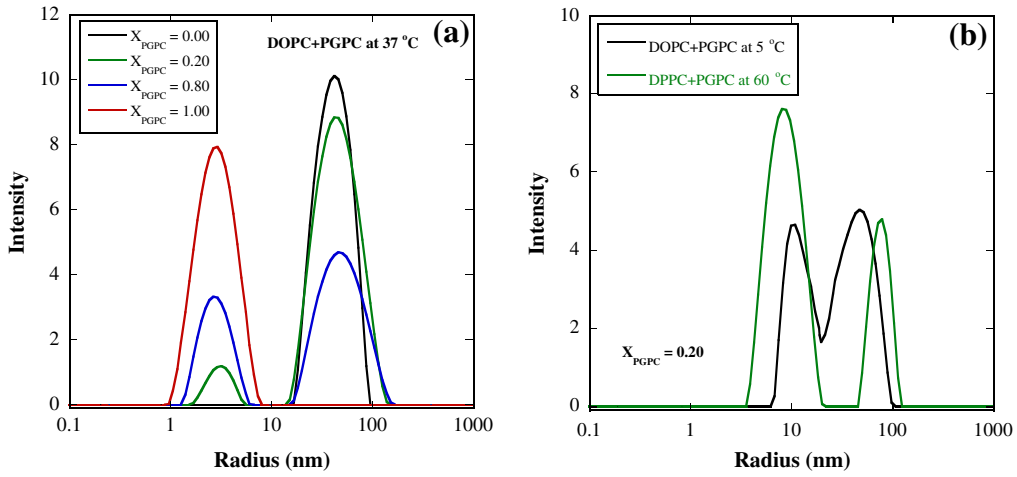

Fig. 5. Dynamic Light Scattering profiles for the hydrodynamic radii of DOPC + PGPC aggregates in the bilayer liquid phase at T = 37 °C. [DOPC] + [PGPC] = 1 mM. (a) Size distribution showing only micelles for PGPC; only vesicles for DOPC; and separation into micelles and vesicles at all mixture compositions: (b) Similarity between the mixing behaviors of PGPC with DPPC at 60 °C and DOPC at 5 °C for $X_{PGPC} = 0.2$ showing equivalence in separation into large micelles and vesicles in both cases. The significance of the two temperatures is that they are both about 20 °C above the respective chain melting transitions.

change in polarity between DOPC/PGPC and DPPC/PGPC at T = 5 °C and 60 °C respectively suggest there is limited mixing that leads to the 9 nm aggregates at temperatures in the liquid phase up to at least 20 °C above the chain melting transition.

3.4. Data summary, reproducibility, and other control experiments

DPPC and DOPC SUV are stable up to at least 72 h. DLS measurements verified that these vesicles did not change size in this time period. DLS and fluorescence measurements on some of the compositions and temperatures (DPPC/PGPC and DOPC/PGPC at 22 °C, DPPC/PGPC at 60 °C and DOPC/PGPC at 5 °C) were repeated on independently prepared samples and also observed over a period of 24 h. The size distributions in the mixtures did not show any change at the end of 24 h. However at 60 °C for DPPC/PGPC and at 5 °C for DOPC/PGPC where the smaller aggregate begins to separately form, the size of the smaller aggregates did change with time; but the larger and smaller aggregates did not merge. The size distributions were also insensitive to whether the mixed samples were simply stirred or sonicated or whether the samples were mixed at the measurement temperature or whether the temperature was raised after mixing at 23 °C. Size distributions were reproducible upon temperature cycling.

The fluorescence experiments on DPPC/PGPC at 22 °C was repeated thrice; DPPC/PGPC at 60 °C and DOPC/PGPC at 22 °C were each repeated twice to confirm the reproducibility of the presence or absence of immediate quenching and slow quenching and the change in I_1/I_3. The data were reproduced to within 6% in the repeated measurements on the selected compositions. For example the average and standard deviation from the three measurements in the quenched fluorescence in DPPC/PGPC at 22 °C for $X_{PGPC} = 0.2$ immediately upon adding PGPC to DPPC was 0.67 ± 0.04 of the unquenched value and I_1/I_3 increased to 1.20 ± 0.02. Each of the data points in Tables 1 and 2 is from one measurement. The mean and standard deviation in the ratio, I_1/I_3 determined from measurements of pyrene fluorescence on eleven independent replicates of pyrene/DPPC and five pyrene/DOPC at 22 °C were 1.05 ± 0.02 and 1.06 ± 0.05 respectively. The change in I_1/I_3, where observed, is well outside the error. Furthermore the supporting evidence for the absence of mixing in the fluid phase, employing the fluorescence quenching assay was gathered not from just one experiment or one temperature but from measurements in the fluid phase at five different temperatures for DOPC and at 60 °C for DPPC.

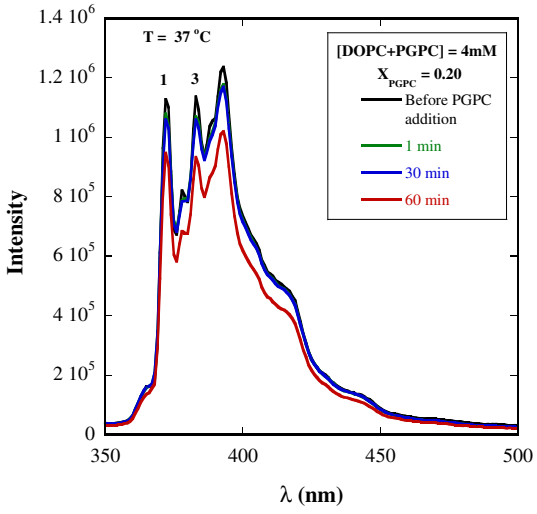

Fig. 6. Fluorescence spectra of pyrene in DOPC bilayers before and after adding PGPC to a final molar fraction of 0.20 PGPC at time intervals, shown in the legend, following addition of PGPC micelles containing DMBP quenchers. T = 37 °C. [DOPC] + [PGPC] = 4 mM. No quenching in the first 1/2 h indicates absence of mixing.

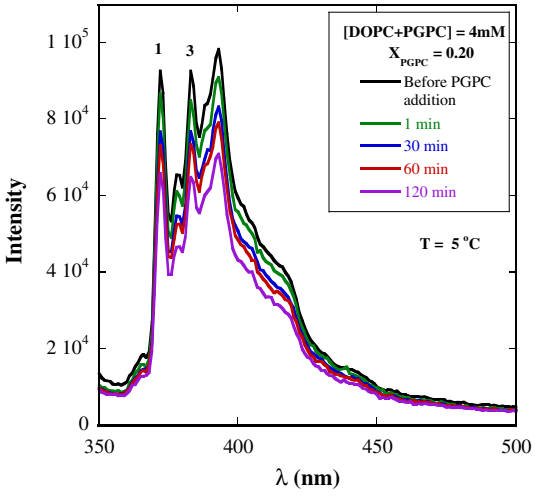

Fig. 7. Fluorescence spectra of pyrene in DOPC bilayers before and after adding PGPC to a final molar fraction of 0.20 PGPC at time intervals, shown in the legend, following addition of PGPC micelles containing DMBP quenchers. T = 5 °C. [DOPC] + [PGPC] = 4 mM. Similarity with Fig. 3c is to be noted.

Table 2
Pyrene fluorescence emission intensity at 393 nm and polarity of pyrene neighborhood, as determined by the ratio I_1/I_3, at various times following addition of PGPC micelles containing DMBP quenchers to DOPC bilayers containing pyrene. The emission intensity before addition is set to 1 with subsequent intensities expressed as fractions thereof.

Time (min)	$X_{PGPC} = 0.20$									
	T = 5 °C		T = 22 °C		T = 37 °C		T = 50 °C		T = 60 °C	
	I_{393}	I_1/I_3	I_{393}	I_1/I_3	I_{393}	I_1/I_3	I_{393}	I_1/I_3	I_{393}	I_1/I_3
Before	1.00	1.00	1.00	1.10	1.00	0.96	1.00	1.05	1.00	1.00
0	0.93	1.00	1.00	1.10	1.00	0.92	1.00	1.01	1.00	1.00
30	0.85	1.00	1.00	1.10	0.94	0.94	1.00	1.01	0.97	1.00
60	0.81	1.00	0.94	1.09	0.88	0.94	0.87	1.02	0.97	1.00
120	0.73	1.00	0.94	1.09	0.82	0.94	0.72	1.03		
180			0.91	1.09	0.46	0.92	0.63	1.04		
240			0.95	1.09	0.45	0.93	0.56	1.06		

The fluorescence quenching assay was designed to detect the uptake of oxidized lipids by bilayers. The three types of temporal behavior of quenching encountered in this work; including immediate quenching, absence of immediate quenching, and slow increase in quenching, are depicted in Fig. 8.

The interpretation that presence and absence of immediate quenching represent presence and absence of fusion is consistent with the DLS data and therefore the most reasonable. When mixing is present, the intensity drops and I_1/I_3 increases (Fig. 8a and c for DPPC/PGPC). Fig. 8a shows clearly the presence of immediate quenching in DPPC gel phase and its absence in DOPC fluid phase. The 22 °C DOPC/PGPC data in Fig. 8a is distinct from the DPPC/PGPC data in the same figure and also from the data of DPPC/PGPC at 60 °C and DOPC/PGPC at 5 °C in Fig. 8b; enough to justifiably be regarded as exhibiting absence of immediate quenching. Lack of immediate quenching together with no increase in I_1/I_3 (Fig. 8 a and c) is interpreted as the effect of the absence of mixing in accordance with DLS results.

In order to aid the interpretation that slow development of quenching, where observed, is because of slow exchange of the hydrophobic probes between aggregates; fluorescence quenching measurements of DOPC/PGPC mixing at 37 °C with about 5 vol.% ethyl alcohol (EtOH, which is known to accelerate exchange) in the solution was conducted. A standard practice for incorporating hydrophobic solutes into micelles or bilayers is to inject alcohol or acetonitrile solutions of these molecules (small amounts of high concentration) into the samples or stir for 24 h when preparing samples using dry films of pyrene (DMBP). Mixing assay was also conducted on samples with DOPC/pyrene and PGPC/DMBP prepared by injecting few microliters of pyrene/EtOH and DMBP/EtOH stock respectively into DOPC and PGPC solutions. Immediate quenching upon adding PGPC with DMBP to DOPC with pyrene was observed in both cases, showing that pyrene and DMBP exchange was enhanced by the presence of alcohol. Exchange via the aqueous medium is a slow process and presence of small amounts of alcohol (1% or even less) enhances the exchange rate. In the absence of any alcohol therefore, the slow development of quenching observed indicates absence of fusion and slow exchange of pyrene and DMBP.

As a reference for the magnitude of the maximum quenching, fluorescence quenching experiments were conducted on premixed DPPC/PGPC and DOPC/PGPC ($X_{PGPC} = 0.2$) vesicles containing pyrene;

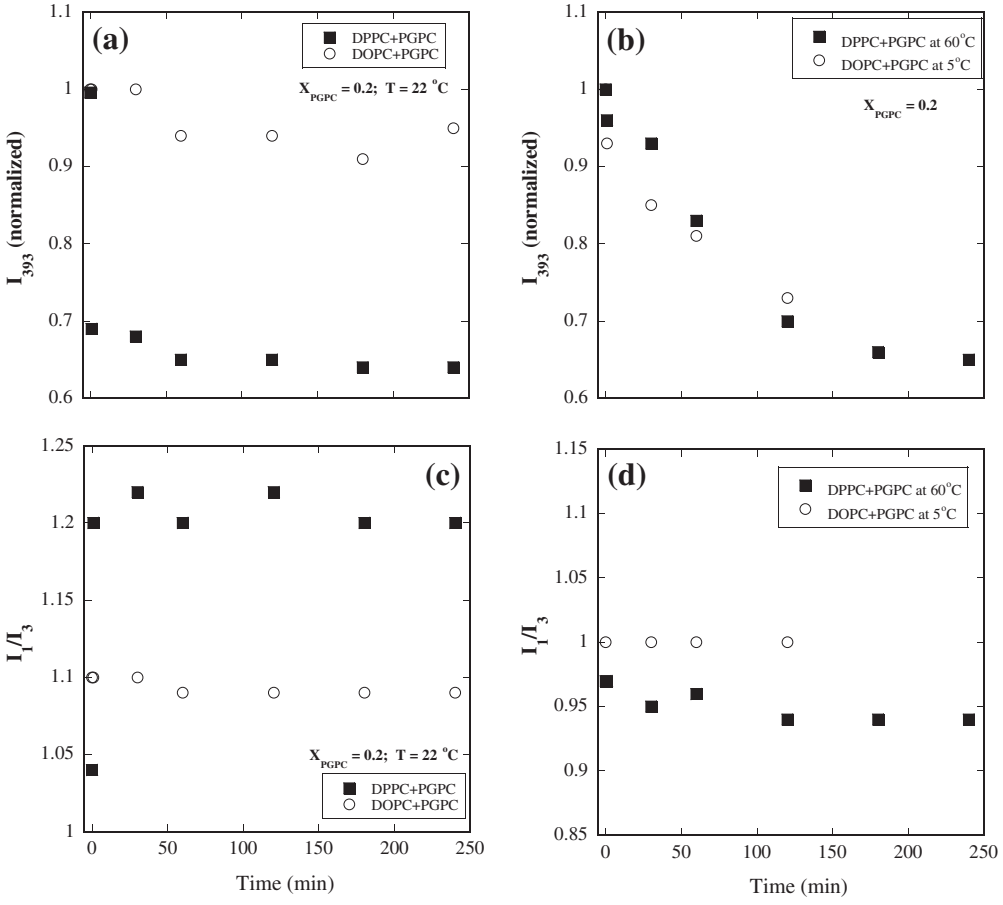

Fig. 8. Graphical display of the three types of temporal behavior of quenching encountered, following addition of quencher containing PGPC micelles to pyrene containing DPPC or DOPC vesicles. These include (a) immediate quenching in the DPPC gel phase, its absence in the DOPC fluid phase and (b) slow quenching in DPPC and DOPC at equivalent temperatures of 20 °C above their respective T_m. (c) Increase in I_1/I_3 accompanies immediate quenching in DPPC/PGPC at 22 °C. In DOPC/PGPC at 22 °C, where quenching is absent, I_1/I_3 continues with its initial value in DOPC. (d) I_1/I_3 is unchanged also when quenching develops slowly.

with and without DMBP. The vesicles were prepared by sonicating hydrated premixed films of DPPC or DOPC and PGPC. Fluorescence intensity decreased to 0.25 of the unquenched value in the vesicles with DMBP. Pyrene and DMBP in the fluorescence assays may not reach the same distribution as in the premixed and sonicated vesicles in the first few hours of adding PGPC to DPPC or DOPC irrespective of whether mixing is present or not. This could be the reason why the quenching observed in the first few hours in DPPC/PGPC or DOPC/PGPC at 22 °C for $X_{PGPC} = 0.2$ is not to the extent found in premixed vesicles. The fluorescence intensity in DPPC/PGPC as well as DOPC/PGPC for $X_{PGPC} = 0.2$ did decrease to 0.3 of the unquenched value in 11 and 24 h respectively while the initial size distributions, observed by DLS, remained the same over the course of 24 h for these compositions. Fusion manifests measureable levels of immediate quenching. In the fluorescence assay experiments, presence or absence of fusion is distinguished by whether there is immediate quenching or not. Development or slow increase in quenching that occurs thereafter and also when there is no fusion, is due to the mechanics of pyrene/DMBP redistribution.

Quenching in DPPC/PGPC at 60 °C and in DOPC/PGPC (Fig. 8b and Table 2) may be viewed simply as slower quenching in the fluid phase compared to that in the gel phase. In order to confirm that the slow quenching in the fluid phase is an effect of absence of mixing rather than that of the nature of the phase, experiments were conducted on DOPC/LOPC (lysooleoylphosphatidylcholine) at $X_{LOPC} = 0.2$ at 22 °C. Aggregates in this mixture were mixed vesicles as measured by DLS. This is different from the coexisting vesicles and micelles in DOPC/PGPC mixtures. Fluorescence quenching assay of mixing was conducted on DOPC/LOPC. Immediate quenching was observed with a drop in intensity to 69% of the unquenched value signifying mixing as opposed to the slow quenching in DOPC/PGPC. DOPC is in the fluid phase in both cases. Therefore the slow quenching in DOPC/PGPC and the immediate quenching in DOPC/LOPC must be due to absence and presence of mixing respectively in agreement with the DLS results. The underlying reason for the slow increase in quenching must be due to pyrene and DMBP present initially in the different aggregates (whose presence is shown by DLS). Pyrene and DMBP are to be expected to redistribute and to not continue to be compartmentalized. Slow rate of transfer during collisions gives rise to slowly changing concentrations.

4. Discussion

The scope of this work was to determine the presence or absence of mixing upon addition of PGPC to DPPC or DOPC first from the size distribution of the ensuing aggregates and then using a fluorescence quenching assay designed to test predicted outcomes of mixing in such experiments. Presence of only vesicles was treated as evidence of mixing. Presence of coexisting small aggregates of the size of PGPC micelles and large aggregates of the size of vesicles is evidence of separation. Information on the basic property that bilayer phospholipids and PGPC fuse to form mixed vesicles only up to a PGPC molar fraction of about 0.3 and only in the gel phase and separate into vesicles and micelles in the liquid phase was made available by the DLS experiments. Complementing with the pyrene fluorescence quenching assay gives further insight into the temperature and phase dependence of the character of the mixed aggregates. DLS is not sensitive to the lipid organization or composition within the mixed vesicle and also no information on vesicle shape is available from these measurements. But this does not affect the conclusions on the presence of mixing or demixing and that vesicles are present in the mixtures. The coexisting bilayer lipid vesicles and PGPC micelles are to be expected to contain small amounts of the other component according to the requirements of entropy.

Stability in the size distribution over the observed period of 24 h; similarity of the size distribution between samples prepared by adding PGPC to DPPC or DOPC followed by stirring and samples prepared by vortexing and sonicating the mixture; and reversibility upon temperature cycling show that equilibrium size distributions are observed in the present experiments. The fluorescence quenching assay by design on the other hand follows the time development in the quenching magnitude and the polarity of pyrene neighborhood following addition of PGPC to DPPC or DOPC bilayers. Immediate quenching with concomitant increase in I_1/I_3 indicating water penetration is to be reasonably expected of fusion and was therefore treated as evidence of fusion. Slow increase in quenching is more likely the effect of the redistribution rate of pyrene and DMBP particularly when probes and quenchers are initially in different aggregates.

Interpretation of the DPPC/PGPC data at T = 50 °C, and the similarity in the behavior of DPPC/PGPC at T = 60 °C to that of DOPC/PGPC at T = 5 °C together with data at temperatures in the gel phase of DPPC and liquid phase of DOPC suggest a pattern in the progression of the aggregation states, from a mixed vesicle in the gel phase to aggregation states with different levels of bilayer lipid-PGPC separation in the liquid phase. Upon increasing the temperature beyond the bilayer phase transition, the mixed vesicle first undergoes lateral separation into bilayer lipids and PGPC or hemi fused micelles and vesicles, followed by successive transitions to coexisting bilayer lipid vesicles and mixed PGPC/bilayer lipid micelles and separated but coexisting bilayer lipid vesicles and PGPC micelles. Experiments on mixtures of POPC and the OxPL, also report presence of lateral separation into PGPC rich and POPC rich domains at T = 5 °C and 10 °C which are within 20 °C of the POPC bilayer melting transition of −2 °C [16]. The observations reported on POPC are illustrative of the first stage of separation, a few degrees above the T_m, in the progression outlined, which at this point is an interpretation consistent with the present DLS and fluorescence quenching assay data and data reported in the literature.

Heterogeneity of lipids present in cell membranes has motivated several studies of lipid organization in fluid bilayers. Mixing between dissimilar lipids is in general non-ideal [21]. Non-ideality is embodied in the free energy difference, ω, between having unlike lipid neighbors and like lipid neighbors. Unfavorable interaction between unlike lipids yields a positive value for the thermodynamic parameter ω, which leads to segregation and domains in mixed lipid bilayers. ω depends on the types of lipids, lipid composition, pH, temperature, and bilayer phase. Examples exist for larger ω values in the fluid phase than in the gel phase and also for positive values in the fluid phase and negative values in the gel phase [21]. In such cases, mixed lipid bilayers exhibit preference for lipid separation in the fluid phase and mixing in the gel phase. In the present case of mixtures of bilayer forming phospholipids and micelle forming lipids, demixing in the fluid phase manifests as lipid separation within the same bilayer or hemi fused micelles and vesicles or separation into vesicles and micelles, depending on the temperature with respect to the melting transition temperature.

Preference for mixing or association in the gel or ordered phase and separation in the fluid phase are found in other phenomena as well. Cholesterol prefers the liquid ordered phase rather than the liquid disordered phase [21,22]. Inclusion of fatty acids and diacylglycerols induces ordering in lipid bilayers, rather than homogeneous mixing [23]. Fusion in the gel phase and its absence in the liquid phase have been observed in inter vesicle fusion as well. Bilayers with an interdigitated arrangement of lipids are open to fusion in the gel phase but not in the liquid phase [18,24].

5. Summary and conclusions

Mixing of the bilayer phospholipids DPPC and DOPC with the micelle forming OxPL, PGPC is non-ideal in the fluid bilayer phase. Mixing behavior was studied through observations of the aggregation states of DPPC/PGPC and DOPC/PGPC in the temperature range 5 to 60 °C. In the gel phase DPPC and PGPC form mixed vesicles only for PGPC molar fractions ≤0.3. For higher molar fractions DPPC and PGPC aggregate separately and coexist as vesicles and micelles in both gel and fluid phases. Only the fluid phase was accessible for investigating

DOPC/PGPC, which showed the same mixing characteristics of non-ideality as DPPC/PGPC in its fluid phase, namely separation into vesicles and PGPC micelles for all compositions. Based on the combined results for DOPC in the fluid phase and DPPC in the gel and fluid phases, a bilayer temperature/phase and composition dependent progression in the aggregation states is indicated. The data suggest that, when $X_{PGPC} \leq 0.3$, aggregation states change progressively from mixed vesicles in the gel phase to mixed vesicles with a lateral separation between the bilayer phospholipid and PGPC in the liquid phase or hemi fused micelles and vesicles close to the bilayer chain melting transition temperature to bilayer lipid and oxidized lipid separated coexisting vesicles and micelles at higher temperatures. DOPC–PGPC and DPPC–PGPC mixing is non-ideal for $X_{PGPC} > 0.3$ in both gel and fluid phases resulting in exclusion of PGPC from the bilayer. The present results contribute toward establishing an organizing principle that mixing of oxidized and unoxidized lipids is favored in the gel phase up to a limited composition but not in the fluid phase for any composition.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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References

[1] R. Salvayre, N. Auge, H. Benoist, A. Negre-Salvayre, Oxidized low-density lipoprotein-induced apoptosis, Biochim. Biophys. Acta 1585 (2002) 213–221.
[2] G.O. Fruhwirth, A. Moumtzi, A. Loidl, E. Ingolic, A. Hermetter, The oxidized phospholipids POVPC and PGPC inhibit growth and induce apoptosis in vascular smooth muscle cells, Biochim. Biophys. Acta 1761 (2006) 1060–1069.
[3] G.O. Fruhwirth, A. Loidl, A. Hermetter, Oxidized phospholipids: from molecular properties to disease, Biochim. Biophys. Acta 1772 (2007) 718–736.
[4] S. Rhode, R. Grurl, M. Brameshuber, A. Hermetter, G.J. SchÜtz, Plasma membrane fluidity affects transient immobilization of oxidized phospholipids in endocytotic sites for subsequent uptake, J. Biol. Chem. 284 (2009) 2258–2265.
[5] E. Gibbons, J. Nelson, L. Anderson, K. Brewer, S. Melchor, A.M. Judd, J.D. Bell, Role of Membrane oxidation in controlling the activity of human group IIa secretory phospholipase A_2 toward apoptotic lymphoma cells, Biochim. Biophys. Acta Biomembr. 1828 (2013) 670–676.
[6] P. Jurkiewicz, A. Olżyńska, L. Cwiklik, E. Conte, P. Jungwirth, F.M. Megli, H. M., Biophysics of lipid bilayers containing oxidatively modified phospholipids: insights from fluorescence and EPR experiments and from MD simulations, Biochim. Biophys. Acta Biomembr. 1818 (2012) 2388–2402.
[7] D. Arrais, J. Martins, Bilaer Polarity and its thermal dependency in the *l*0 and *l*d phases of binary phosphatidylcholine/cholesteorol mixtures, Biochim. Biophys. Acta Biomembr. 1768 (2007) 2914–2922.
[8] M.S. Bakshi, J. Singh, G. Kaur, Fluorescence study of solubilization of l-α-dilauroylphosphatidylethanolamine in the mixed micelles with monomeric and dimeric cationic surfactants, J. Photochem. Photobiol. A Chem. 173 (2005) 202–210.
[9] P.J. Wyatt, Light scattering and the absolute characterization of macromolecules, Anal. Chim. Acta 272 (1993) 1–40.
[10] M. Tachiya, Application of a generating function to reaction kinetics in micelles. Kinetics of quenching of luminescent probes in micelles, Chem. Phys. Lett. 33 (1975) 289–292.
[11] M. Tachiya, On the kinetics of luminescence quenching in micellar systems, J. Chem. Phys. 78 (1983) 5282.
[12] M.H. Gehlen, F.C. De Schryver, Time-resolved fluorescence quenching in micellar assemblies, Chem. Rev. 93 (1) (1993) 199–221.
[13] J. Singh, J. Miller, R. Ranganathan, Physicochemical characterization of phospholipid solubilized mixed micelles and a hydrodynamic model of interfacial fluorescence quenching, J. Phys. Chem. B 111 (31) (2007) 9317–9324.
[14] J. Singh, Z. Unlu, R. Ranganathan, P.C. Griffiths, Aggregate properties of sodium deoxycholate and dimyristoylphosphatidylcholine mixed micelles, J. Phys. Chem. B 112 (13) (2008) 3997–4008.
[15] Y. Li, V. Lubchenko, P.G. Vekilov, The use of dynamic light scattering and Brownian microscopy to characterize protein aggregation, Rev. Sci. Instrum. 82 (2011) (053106-053101-053108).
[16] L. Beranová, L. Cwiklik, P.-. Jurkiewicz, M. Hof, P. Jungwirth, Oxidation changes physical properties of phospholipid bilayers: fluorescence spectroscopy and molecular simulations, Langmuir 26 (2010) 6140–6144.
[17] A. Almgren, Migration and partitioning of pyrene and perylene between lipid vesicles in aqueous solution studied with a fluorescence stopped-flow technique, J. Am. Chem. Soc. 102 (1980) 7882–7887.
[18] J.H.K. Rozenfeld, E.L. Duarte, J.-M.L. Ruysschaert, C. Lonez, M.T. Lamy, Structural characterization of novel cationic diC16-amidine bilayers: evidence for partial interdigitation, Biochim. Biophys. Acta Biomembr. 1848 (2015) 127–133.
[19] C. Bohne, R. Konuk, J.C. Scaiano, Dynamics of the redistribution of 1-dodecylpyrene aggregates in micellar solution, Chem. Phys. Lett. 152 (1988) 156–159.
[20] E. Melo, A.A. Freitas, C. Yihwa, F.H. Quina, On the significance of the solubilization power of detergents, Langmuir 17 (2001) 7980–7981.
[21] P.F.F. Almeida, Thermodynamics of lipid interactions, Biochim. Biophys. Acta 1768 (2009) 72–85.
[22] M.R. Krause, T.A. Daly, P.F.F. Almeida, S.L. Regen, Push–pull mechanism for lipid raft formation, Langmuir 30 (2014) 3285–3289.
[23] F.M. Harris, S.K. Smith, J.D. Bell, Physical properties of erythrocyte that determine susceptibility to secretory phospholipase A_2, J. Biol. Chem. 276 (2001) 22722–22731.
[24] H. Komatsu, S. Okada, Ethanol-induced aggregation and fusion of small phosphatidylcholine liposome: participation of interdigitated membrane formation in their processes, Biochim. Biophys. Acta 1235 (1995) 270–280.