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Mixing of oxidized and bilayer phospholipids

Jasmeet Singh, Radha Ranganathan *

Department of Physics and Center for Supramolecular Studies, California State University (CSU), Northridge, CA 91330-8268, USA

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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

* Corresponding author. Tel.: +1 818 677 2942.
E-mail address: Radha.ranganathan@csun.ed (R. Ranganathan).

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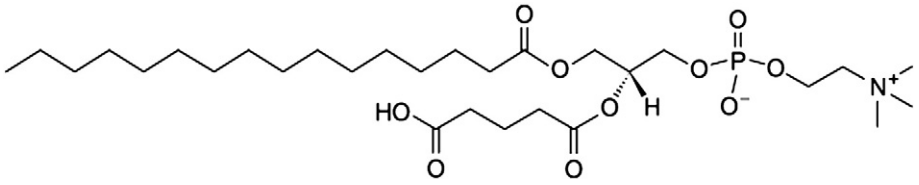

Fig. 1. Chemical structure of the oxidized phospholipid, PGPC.

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