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The Chemical Nature of the Polar Functional Group of Oxidized Acyl Chain Uniquely Modifies the Physicochemical Properties of Oxidized Phospholipid-Containing Lipid Particles

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Abstract Oxidative modification of phospholipids generates a variety of oxidized phospholipid (Ox-PL) species which differ considerably in their chemical compositions and molecular structures. Recent results suggest that even closely related Ox-PL species can have considerably different biological effects. However, the molecular mechanism for this is not yet clear. In truncated Ox-PLs (tOx-PLs) the fatty acyl chain is shorter in length than the parent nonoxidized phospholipid molecules and contains a polar functional group(s). In a previous study we showed that two closely related tOx-PL species having a similar polar functional group and differing only in the length of the oxidized fatty acyl chain exerts significantly different effects on the physicochemical properties of the nonoxidized phospholipid particles containing these lipids (Kar et al., Chem Phys Lipids 164:54–61, 2011). In this study we have characterized the effect of polar functional groups of oxidized fatty acyl chain on the physicochemical properties of the nonoxidized phospholipid particles containing these lipids. Our results show that Ox-PL species differing only in the chemical nature of polar functional groups in their oxidized fatty acyl chain modify the properties of nonoxidized phospholipid particles containing them in a distinctive way. These results indicate that different species of Ox-PLs induce unique changes in the physicochemical properties of lipid particles/membranes containing them and that this may lead to their different biological effects.

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Introduction

Oxidative modifications of lipids are part of normal biological processes and turnover. However, during certain pathological conditions, such as chronic inflammation and other vascular diseases, oxidative stress is more enhanced. Oxidation of phospholipids results in generation of a plethora of oxidized phospholipid (Ox-PL) species which differ considerably in their molecular structures, chemical compositions, physicochemical properties and biological effects (Bochkov et al. 2010; Catalá 2009; Fruhwirth et al. 2007; Fu and Birukov 2009). These oxidized products are involved in a variety of pathological and physiological functions (Catalá 2009; Deigner and Hermetter 2008; Fruhwirth et al. 2007; Fu and Birukov 2009). Under physiological conditions in the body, oxidation of lipids is mediated by both enzymatic as well as nonenzymatic mechanisms. Generation of different types of Ox-PL species depends on the chain length and number and the position of double bonds in the fatty acyl chain of the phospholipid molecules. Different types of Ox-PL species include oxygenated phospholipids, truncated Ox-PLs (tOx-PLs) and other low-molecular weight oxidized species (Bochkov et al. 2010; Catalá 2009; Fruhwirth et al. 2007; Fu and Birukov 2009; Levitan et al. 2010).

Numerous reports indicate that the biological effects of closely related Ox-PL species differ considerably (Subbanagounder et al. 2000; Leitinger et al. 1999; Miller et al. 2011; Fruhwirth et al. 2007; Fu and Birukov 2009; Johnstone et al. 2009). However, the reason for the differential effects of closely related Ox-PL species is not clear. We

believe that due to their unique chemical compositions, individual Ox-PL species possess distinctive properties and that the presence of particular Ox-PL species modifies the overall physicochemical properties of lipid particles containing them in a typical way. To prove this we have characterized the properties of lipid particles containing particular types of closely related Ox-PL species. In our previous studies we have shown that (1) aggregates of pure Ox-PL species possess a distinctive set of physicochemical properties (Pande et al. 2010) and (2) a difference in the length of oxidized fatty acyl chain of Ox-PL species distinctly alters the properties of nonoxidized phospholipid particles containing them (Kar et al. 2011). In this study we show the importance of polar functional groups of oxidized fatty acyl chain in tOx-PLs in modulating the physicochemical properties of nonoxidized phospholipid particles containing them. Lipid particles made up of 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (MPPC) containing varying amounts of either 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC) or 1-palmitoyl-2-(5′-oxo-valeroyl)-*sn*-glycero-3-phosphocholine (POVPC) were prepared and their physicochemical properties characterized. Our results show that the presence of tOx-PLs that differ only in the chemical nature of the polar functional group in their oxidized fatty acyl chains exerts considerably different effects on the physicochemical properties of nonoxidized lipid particles containing them.

Materials and Methods

Materials

MPPC and 7-nitrobenz-2-oxa-1,3-diazol-4-yl-1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (NBD-DMPG) were purchased from Avanti Polar Lipids (Alabaster, AL). PGPC and POVPC were from Cayman Chemical (Ann Arbor, MI). The fluorescent probes *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (F-DHPE) and 2-dimethylamino-(6-lauroyl)-naphthalene (Laurdan) were from Molecular Probes (Invitrogen, Bangalore, India). Deuterium oxide (99.9 % pure) was obtained from Sigma-Aldrich (Bangalore, India). All other reagents were of analytical grade and purchased from Sigma-Aldrich. Double-deionized water was used for buffer preparation.

Preparation of Phospholipid Particles Containing Varying Amounts of Ox-PL Species

Lipid particles containing MPPC (nonoxidized phospholipid) and varying amounts of either POVPC or PGPC (tOx-PLs) were prepared in HEPES buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.4), essentially as described previously (Kar et al. 2011). For attenuated total reflection Fourier transform infrared (ATR-FTIR) experiments D_2O-based buffer was used. Appropriate amounts of lipid stock solutions in chloroform or ethanol were mixed to obtain the desired lipid molar ratio. After removing the organic solvent, the dry film was hydrated by adding an appropriate amount of HEPES buffer and vortexing the samples at room temperature. To label the lipid particles with fluorescent probes, an appropriate amount of fluorescent dye was then added to the sample to attain the desired probe:lipid molar ratio. NBD-DMPG (0.1 mol%)-labeled phospholipid particles, used in the electrophoresis, were prepared by adding an appropriate amount of NBD-DMPG from the stock to lipid mixtures, followed by the removal of solvent under nitrogen, desiccation under vacuum and finally rehydration of dry film at room temperature by adding an aqueous buffer as described above. The lipid mixtures were then incubated at temperatures above and below the main phase transition temperature (T_m) of MPPC (35 ± 1 °C) (Huang and Mason 1986), i.e., 10 min at 45 °C, followed by 10 min at 25 °C. After three temperature transition cycles, the mixtures were finally incubated overnight at 35 °C.

Fluorescence Spectroscopy

All fluorescence measurements were made using a Shimadzu spectrofluorophotometer (RF-5301PC; Shimadzu, Kyoto, Japan), as described earlier (Kar et al. 2011). Briefly, the temperature of the cuvette holding the sample was maintained using a constant temperature water circulator (NTT-2200P), and the sample in the cuvette was stirred constantly with a stirrer. Excitation and emission slits were kept at 5 and 10 nm, respectively. All spectra were corrected by subtracting the spectra of buffer or the spectra of phospholipid suspension without fluorescent probe and smoothed (Savitzky-Golay, 11 points).

Effect of tOx-PLs on the Surface Properties of the Lipid Particles

The effect of tOx-PL species on the surface properties of the lipid particles was determined by monitoring the fluorescence emission spectra of F-DHPE (Kar et al. 2011). In fluorescein-DHPE, the green-fluorescent, pH-sensitive fluorescein is attached on the headgroup of DHPE; and when this probe gets incorporated into lipid bilayers, fluorescein locates itself on the membrane surface and provides information on the surface properties of the lipid vesicles (Soucaille et al. 1988; Kubica et al. 2003). Lipid particles (0.5 ml, 300 μM final concentrations) containing 0.1 mol% F-DHPE were prepared, and the fluorescence

emission spectrum of F-DHPE was recorded from 500 to 600 nm using an excitation wavelength of 480 nm. The relative changes in the fluorescence intensities of the membrane incorporated F-DHPE were calculated using the following formula (Kar et al. 2011):

$$\text{Change in fluorescence intensities} = (F - F_o)/F_o \qquad (1)$$

where F_o is the F-DHPE fluorescence intensity when the phospholipid particle does not contain tOx-PL and F is the fluorescence intensity of a particle containing the stated concentration of tOx-PL.

Effect of tOx-PL on the Acyl Chain Order in the Lipid Particles

The effect of tOx-PL species or increasing temperature on the order of fatty acyl chains in the lipid particles was determined using Laurdan as a fluorescent probe, as described previously (Kar et al. 2011; Pande and Tillu 2011). It is known that, when excited at 360 nm, the emission maxima of Laurdan shifts to a longer wavelength with increasing acyl chain disorder in the phospholipid membrane, resulting in a decrease in the generalized polarization (GP) values (Parasassi et al. 1991). Lipid particles labeled with 1 mol% Laurdan (500 µl, 300 µM final concentrations) were incubated in the cuvette at 25 °C or at designated temperatures. 3 min after incubation fluorescence emission spectra were recorded from 400 to 500 nm using an excitation wavelength of 360 nm. GP values for Laurdan, which reflects fatty acyl chain order, were calculated from emission spectra using the following equation (Parasassi et al. 1991):

$$\text{GP} = (F_{440} - F_{490})/(F_{440} + F_{490}), \qquad (2)$$

where F_{440} and F_{490} are the fluorescence emission intensities at 440 and 490 nm, respectively.

Attenuated Total Reflection Fourier Transform Infrared Spectroscopy

ATR-FTIR spectra of the samples were recorded on a Tensor 27 FTIR spectrometer (Bruker Optics, Ettlingen, Germany). The instrument was equipped with a liquid, N_2-cooled mercury–cadmium–telluride detector, a BioATRCell^{TM} I containing a ZnSe crystal as an internal reflective element and a software-operated polarizer (aluminum grid on KRS-5 substrate, Bruker Optics). The spectrophotometer was constantly purged with dry air. Spectra recording and data processing were done using OPUS software (Bruker Optics). Lipid particle suspensions in D_2O-based buffer (100 µl of 5 mM) were deposited on the surface of the ZnSe crystal of the BioATRCell^{TM} I, and thin, semidried film was prepared by slow evaporation of the samples under a gentle stream of N_2 gas. This method of sample preparation is known to yield a well-ordered multilayer stack of sample molecules (Goormaghtigh et al. 1999; Tatulian 2003). The chamber containing the BioATRCell^{TM} I was then sealed, and the spectra were recorded at 25 °C. For each sample 526 interferograms were accumulated to improve the signal/noise ratio at a spectral resolution of 2 cm^{-1}. Spectra were baseline-corrected by rubberband corrections (OPUS software), and the baseline-corrected spectra were used to determine the peak positions as described previously (Kar et al. 2012).

Native PAGE Analysis

Native PAGE analysis was done to determine the effect of tOx-PLs on the molecular size of the lipid particles, as described previously (Kar et al. 2011; Pande et al. 2010). Briefly, equal amounts of 0.1 mol% NBD-DMPG-labeled phospholipid particle suspensions (1.75 mM final concentration) were diluted with 2 × native gel sample buffer and loaded onto a 4–20 % Tris–glycine gel. Electrophoresis was performed using a miniVE electrophoresis unit (GE Healthcare Bio-Sciences, Uppsala, Sweden) at constant voltage. After electrophoresis, lipid particles in the gel were directly imaged using a Typhoon Trio^{TM} Variable Mode Imager (GE Healthcare Bio-Sciences) using an excitation wavelength of 488 nm (blue laser) and an emission wavelength of 520 nm, bandpass 40 filter.

Agarose Gel Electrophoretic Analysis

The effect of Ox-PL content on the surface potential of lipid particles was studied by agarose gel electrophoresis, as described in Sparks and Phillips (1992). Briefly, 0.1 mol% NBD-DMPG-labeled lipid particles were loaded on a 10-cm agarose gel (0.5 %). Electrophoresis was carried out at constant voltage (180 V) in 50 mM Tris–acetate buffer (pH 8.6) for 1 h or until the dye reached the other end. After electrophoresis, bands were observed using the Typhoon Trio Variable Mode Imager.

Results

In this report we have characterized the effect of two tOx-PL species, which differ only in the chemistry of the polar functional group of their oxidized fatty acyl chain, on the physicochemical properties of lipid particles containing them. For this we used two closely related and most studied tOx-PL species: POVPC and PGPC. Both of these tOx-PLs contain a 16-carbon, saturated fatty acyl chain (palmitic acid) at the *sn*-1 position and a 5-carbon saturated acyl chain at the *sn*-2 position and differ only in the chemical

nature of the polar functional group at the *sn*-2 position (Fig. 1). MPPC was used as a model membrane nonoxidized phospholipid for two reasons: (1) it has a gel to liquid crystalline T_m of 35 ± 1 °C, which is much closer to the physiological temperature of 37 °C, and (2) it contains 14-carbon (myristic) and 16-carbon (palmitic acid) saturated fatty acyl chains at the *sn*-1 and *sn*-2 positions, respectively, and most of the phospholipids found in the biological membranes and the lipoprotein particles contain fatty acyl chains of different lengths attached on a glycerol backbone (Davis and Keough 1985). Thus, we believe that, to study the effect of tOx-PLs on the bilayer properties, the MPPC bilayer system is a more physiologically relevant model than the DMPC or DPPC bilayer system.

Fluorescent probes were used to characterize the effect of tOx-PLs on two important properties of lipid particles: changes in the surface properties of lipid particles were determined using F-DHPE as a fluorescent probe (Kar et al. 2011) and changes in the acyl chain order of lipid particles were determined using Laurdan as a probe (Kar et al. 2011; Pande and Tillu 2011). Earlier, we and others showed that these properties of phospholipid particles play important roles in their biological effects (Mattila et al. 2008; Pande et al. 2005, 2009; Pande and Tripathy 2009; Sanchez et al. 2010).

Characterization of Pure MPPC Particles Using F-DHPE and Laurdan as Probes

The effect of lipid phases of MPPC on the fluorescence properties of two probes used in this study has not been characterized earlier. First, we studied the effect of a particular phase (gel, liquid crystalline, etc.) of the MPPC bilayer on the fluorescence emission properties of the probes. To do this an aqueous suspension of fluorescently labeled MPPC particles was subjected to different temperatures and the fluorescence emission spectra were recorded. Figure 2a shows the fluorescence emission spectra of 0.1 mol% F-DHPE incorporated MPPC particles as a function of temperature. An increase in temperature from 22 °C resulted in a decrease in the fluorescence emission intensity of the probe, and at higher temperatures the intensity reached the minimum and remained at the low level. A plot of relative changes in the fluorescence intensities of F-DHPE as a function of temperature (Fig. 2b) clearly indicates that the decrease in the intensity was sharp until ~35 °C (indicated by an arrow) and that after that there was very little change in the intensity.

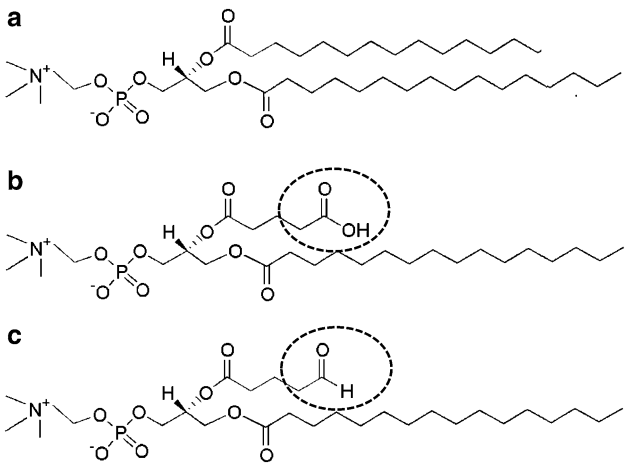

Fig. 1 Structure of phospholipids used in this study. (**a**) MPPC, (**b**) PGPC and (**c**) POVPC

At temperatures much below the T_m, saturated long chain phosphatidylcholines exist in a gel phase. Above T_m the lipid bilayers adopt a liquid-crystalline phase, which is characterized by increased surface area per lipid molecule and completely "melted" hydrocarbon chains (Nagle 1980; Taylor and Morris 1995). The fluorescence properties of F-DHPE depend on the bulk pH, the organization of the lipid's headgroups, the local electrostatic potential in the vicinity of the membrane surface and changes due to variations in the electrostatic dipole orientation, the depth and extent of water penetration and the presence of ions (Kubica et al. 2003). Thus, more penetration of water molecules in the interfacial region of the MPPC bilayer, due to mobilization of the polar headgroup during temperature-induced phase transition, decreases the emission intensity of F-DHPE.

The effect of a particular phase (gel, liquid-crystalline, etc.) of the MPPC bilayer on the fluorescence emission properties of Laurdan was studied by subjecting 1 mol% Laurdan incorporated MPPC particles to increasing temperature (Fig. 2c). An increase in temperature from 22 °C resulted in a decrease in the fluorescence emission intensities along with the shift in the emission maxima from 440 to 490 nm. This typical temperature-dependent red spectral shift is attributed to the dipolar relaxation phenomenon, which is due to the sensitivity of Laurdan to the polarity of its environment (Parasassi et al. 1997). The GP value for Laurdan was calculated from the emission spectra using Eq. 2 and plotted as a function of temperature (Fig. 2d). An increase in temperature resulted in a decrease in the GP values, and at the MPPC phase transition temperature (T_m = 35 °C, indicated by an arrow) the change was very sharp.

Characterization of the temperature-induced phase transition of MPPC particles using F-DHPE and Laurdan suggests that during the temperature-induced phase transition, modification occurred both at the polar surface region as well as at the hydrophobic interior of the lipid bilayer. A similar trend was reported earlier with

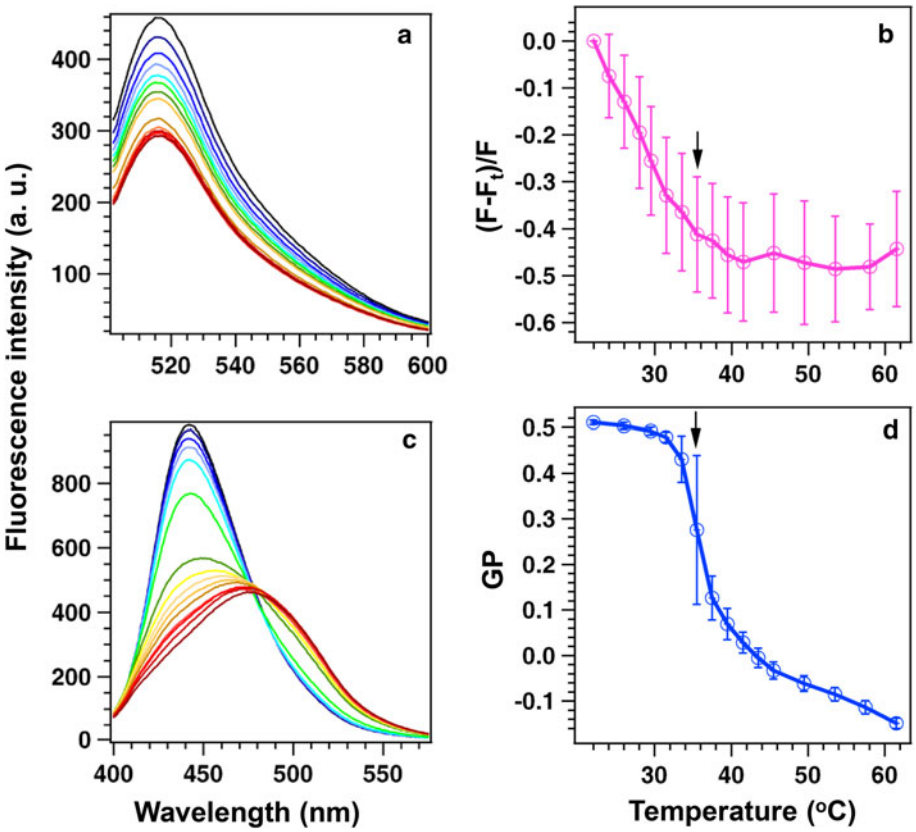

Fig. 2 Temperature-dependent fluorescence emission spectra of (**a**) 0.1 mol% F-DHPE labeled and (**c**) 1 mol% Laurdan-labeled lipid particles made up of 100 % MPPC. An aqueous suspension of 300 μM lipid particles labeled with fluorescent probe was incubated in the cuvette at different temperatures, and the fluorescence emission spectra of F-DHPE and Laurdan were recorded using excitation wavelengths of 490 and 360 nm, respectively. Emission spectra in (**a**) and (**c**) from dark blue to dark red correspond to spectra recorded by increasing temperature from 22 to 62 °C. The relative changes in the fluorescence intensities of F-DHPE (**b**) and the generalized polarization of Laurdan (**d**) were calculated and plotted as a function of temperature. Each data point represents the average of three independent experiments and is expressed as mean ± SEM. *Black arrows* indicate the T_m of MPPC (~35 °C)

phosphatidylcholine-containing fatty acyl chains of comparable length (Kubica et al. 2003).

Effect of tOx-PLs on the Surface Properties of Lipid Particles

The effect of tOx-PL content on the surface properties of lipid particles was determined by monitoring the fluorescence emission spectra of F-DHPE. F-DHPE incorporated (0.1 mol%) lipid particles containing increasing concentrations of PGPC or POVPC were prepared, and the fluorescence emission spectra were recorded at 25 °C (Fig. 3a, b). Compared to lipid particles containing 100 % MPPC, an increase in concentrations of either PGPC or POVPC in lipid particles resulted in a decrease in the emission intensities of the probe. Since the organization of the lipid headgroups and the surface charge density of the lipid particles are known to affect the emission intensity of F-DHPE, the results suggest that an increase in the concentration of either PGPC or POVPC results in a change in the conformation and orientations of lipid headgroups or alteration in the surface charge density. In order to compare the effect, fluorescence emission intensities at 530 nm were used to determine the relative change and are presented in Fig. 3c. At 50 and 75 mol% of tOx-PL, compared to POVPC-containing lipid particles, the relative changes in the emission intensities of the probe were more in PGPC-containing lipid particles. It is interesting to note here that dramatic changes in the membrane containing higher concentrations of Ox-PL in the host lipid were observed in a recent study (Lis et al. 2011). It is interesting to note here that at the *sn*-2 positions, while PGPC contains a 5-carbon fatty acyl chain carrying an ω-carboxyl group, POVPC contains a 5-carbon fatty acyl chain carrying an ω-aldehydic group. In aqueous solution and at neutral pH, the ω-aldehydic and ω-carboxylic groups are zwitterionic and anionic in nature, respectively. The observed effect suggests that the presence of PGPC causes an increase in the surface charge of the lipid particles compared to POVPC. However, the possibility of induction of a conformational

change at the polar headgroup region without increasing the surface charge, as has been observed in the temperature-induced transition of pure MPPC particles, thereby causing a decrease in the emission intensity of F-DHPE, cannot be excluded. In order to prove that the presence of PGPC affects the surface charge of lipid particles, surface charge characteristics of lipid particles containing particular tOx-PLs were determined using agarose gel electrophoresis (discussed below).

Effect of tOx-PL on the Acyl Chain Order in Lipid Particles

The effect of tOx-PL content on the fatty acyl chain order in lipid particles was studied by recording the emission spectra of 1 mol% Laurdan incorporated in lipid particles containing increasing concentrations of either PGPC or POVPC. Normalized fluorescence emission spectra of Laurdan are presented in Fig. 4a, b. At 25 °C MPPC exists in gel phase and fluorescence emission spectra of Laurdan in lipid particles made up of 100 % MPPC exhibit a symmetrical spectrum with an emission maximum at 440 nm. An increase in the concentration of either PGPC or POVPC in the lipid particles resulted in a red shift in the emission maximum of Laurdan. GP values for Laurdan were calculated from the emission spectra using Eq. 2 and plotted as a function of tOx-PL concentration (Fig. 4c). Compared to lipid particles made up of 100 % MPPC, increasing concentrations of tOx-PL in lipid particles resulted in a decrease in GP values; and the extent of the decrease was nearly same in both PGPC- and POVPC-containing lipid particles. Fluorescence emission of Laurdan is known to be sensitive to the presence and mobility of water molecules trapped within the lipid bilayers (Parasassi et al. 1991, 1997). Thus, a decrease in GP values of lipid particles containing increasing concentrations of tOx-PL indicates a decrease in the fatty acyl chain order of lipid particles resulting in more penetration of water into the lipid bilayer. These observations suggest that the presence of either PGPC or POVPC in lipid particles perturbs the hydrophobic interior of the lipid bilayer to a similar extent and are consistent with the ATR-FTIR data (given below).

Lipid Acyl Chain Order Determination by ATR-FTIR Spectroscopy

In order to see the effect of tOx-PL content on the packing of acyl chains in lipid particles, we first determined the lipid acyl chain order by recording the nonpolarized ATR-FTIR spectra of lipid particles. Absorbance of the peaks corresponding to the asymmetric $\nu_{as}(CH_2)$ (2,920 cm^{-1}) and symmetric $\nu_s(CH_2)$ (2,850 cm^{-1}) stretching bands is known to be sensitive to intramolecular vibrational coupling and, thus, provides information on the lateral packing of the hydrocarbon chains in the lipid aggregates (Tamm and Tatulian 1997; Goormaghtigh et al. 1999; Bensikaddour et al. 2008). Absorption maxima of the stretching bands shift to higher and lower vibrational frequencies when lipids are in liquid-crystalline and gel phases, respectively (Tamm and Tatulian 1997). Lipid particles containing varying concentrations of either PGPC or POVPC were prepared and the ATR-FTIR spectra recorded at 25 °C (Fig. 5a, b). Compared to lipid particles containing 100 % MPPC, an increase in the concentration of either PGPC or POVPC in lipid particles resulted in a shift of both asymmetric $\nu_{as}(CH_2)$ and symmetric $\nu_s(CH_2)$ stretching band maxima toward higher frequencies. This clearly indicates that increasing concentrations of tOx-PL

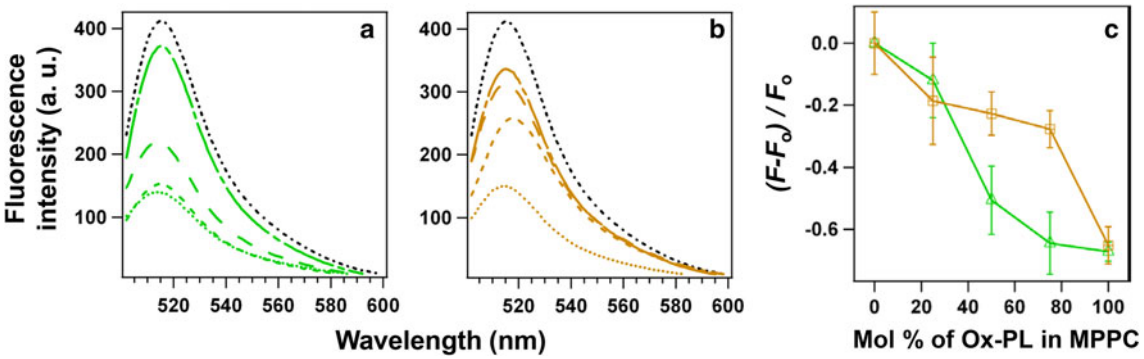

Fig. 3 Representative fluorescence emission spectra of 0.1 mol% F-DHPE incorporated lipid particles containing increasing concentrations of PGPC (**a**) and POVPC (**b**). Fluorescence emission spectra of lipid particle suspension (0.5 ml, 300 μM) were recorded at 25 °C from 500 to 600 nm using the excitation wavelength of 490 nm. *Thick dotted line* 100 % MPPC, *dashed dotted line* 75 % MPPC + 25 % tOx-PL, *long dashed line* 50 % MPPC + 50 % tOx-PL, *dashed line* 25 % MPPC + 75 % tOx-PL, *thin dotted line* 100 % tOx-PL. Values at 530 nm were used to calculate the relative changes in the fluorescence intensities of F-DHPE and plotted as a function of tOx-PL concentrations (**c**). *Triangle* and *square* represent PGPC and POVPC, respectively. Each data point represents the average of three independent experiments and is expressed as mean ± SEM

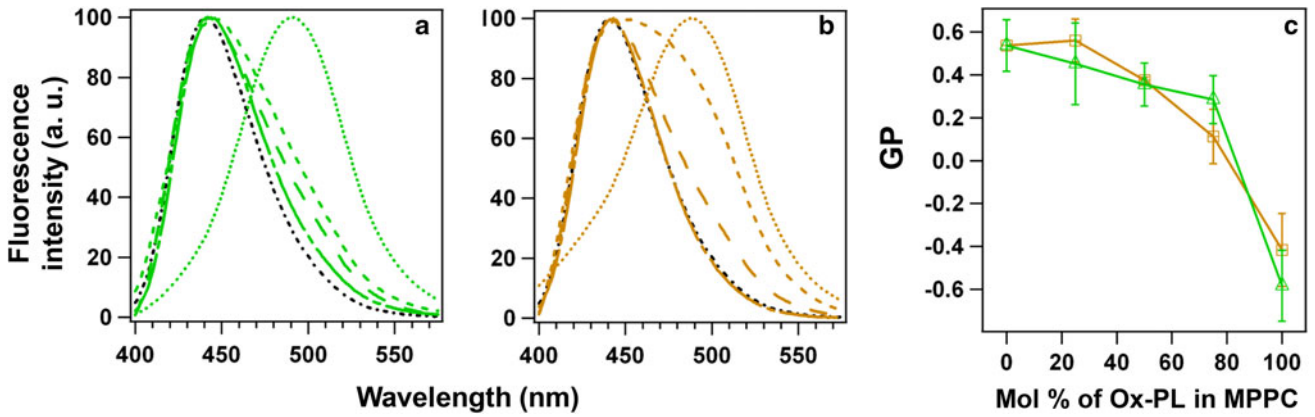

Fig. 4 Normalized fluorescence emission spectra of 1 mol% Laurdan incorporated lipid particles containing increasing concentrations of PGPC (**a**) and POVPC (**b**). Fluorescence emission spectra of phospholipid particle suspension (0.5 ml, 300 μM) were recorded at 25 °C from 400 nm to 600 nm by exciting the Laurdan at 490 nm. *Thick dotted line* 100 % MPPC, *dashed dotted line* 75 % MPPC + 25 % tOx-PL, *long dashed line* 50 % MPPC + 50 % tOx-PL, *dashed line* 25 % MPPC + 75 % tOx-PL, *thin dotted line* 100 % tOx-PL. Emission intensities at 440 and 490 nm were used to calculate the GP of Laurdan and plotted as a function of tOx-PL concentrations (**c**). *Triangle* and *square* represent PGPC and POVPC, respectively. Each data point represents the average of three independent experiments and is expressed as mean ± SEM

affect the packing of the acyl chain in lipid particles and increase the fluidity of lipid particles. A plot of asymmetric $\nu_{as}(CH_2)$ vibration maxima as a function of tOx-PL concentrations is shown in Fig. 5c. The results suggest that both PGPC and POVPC perturb the hydrophobic interior of the lipid bilayer and decrease the fatty acyl chain order in lipid particles to a similar extent.

Effect of tOx-PL on the Molecular Size of Lipid Particles by Native PAGE Analysis

Native PAGE analysis is a predominant technique used to determine the molecular size and relative homogeneity of the lipid and lipoprotein particles (Kar et al. 2011; Pande et al. 2010). To determine the effect of closely related tOx-PLs on the molecular size and homogeneity of lipid particles, 0.1 mol% NBD-DMPG incorporated lipid particles containing different concentrations of either PGPC or POVPC were made and subjected to native PAGE analysis on a 4–20 % gradient gel. A representative gel image is shown in Fig. 6a. Lipid particles were observed as a band (pseudoyellow) on the gel, suggesting the formation of lipid particles. The same types of homogenous lipid particles were observed when the mixture of DMPC with either PGPC or POVPC or PazePC or PoxnoPC was analyzed by native PAGE analysis (data not shown). Because of its large size and zwitterionic nature, an aqueous suspension of 100 % MPPC particles, which forms multilamellar vesicles, showed a band that failed to enter the gel and remained at the bottom of the loading well (Fig. 6, lane a). Increasing concentrations of tOx-PLs resulted in formation of lipid particles of different sizes (Fig. 6, lanes b–i). Thus, native PAGE analysis indicates that the relative molecular size of lipid particles containing PGPC or POVPC differs considerably and depends on the concentrations of tOx-PL present.

Effect of tOx-PL on the Surface Potential of Lipid Particles by Agarose Gel Electrophoresis

The effect of tOx-PL content on the surface potential of lipid particles was determined by subjecting 0.1 mol% NBD-DMPG-labeled MPPC particles containing different concentrations of either PGPC or POVPC to agarose gel electrophoresis. A representative gel image is presented in Fig. 7. Phospholipid particles were detected as a large band (pseudoyellow) on the agarose gel. An aqueous suspension of 100 % MPPC particles, which forms multilamellar vesicles, showed a band that failed to enter the gel and remained at the bottom of the loading well, because of its large size and lack of a net charge (Fig. 7, lane a). Increasing the concentration of POVPC and PGPC resulted in the formation of phospholipid particles, which migrated differentially on the agarose gel. The results suggest that closely related tOx-PLs exert significantly different effects on the surface charge properties of lipid particles containing them.

Discussion

Ox-PLs differ considerably in their properties, and closely related Ox-PL species have been shown to interact differently with proteins and target lipid membranes and to exert considerably different biological effects (Leitinger et al. 1999; Johnstone et al. 2009; Beranova et al. 2010; Fruhwirth et al. 2007; Fu and Birukov 2009; Greenberg et al.

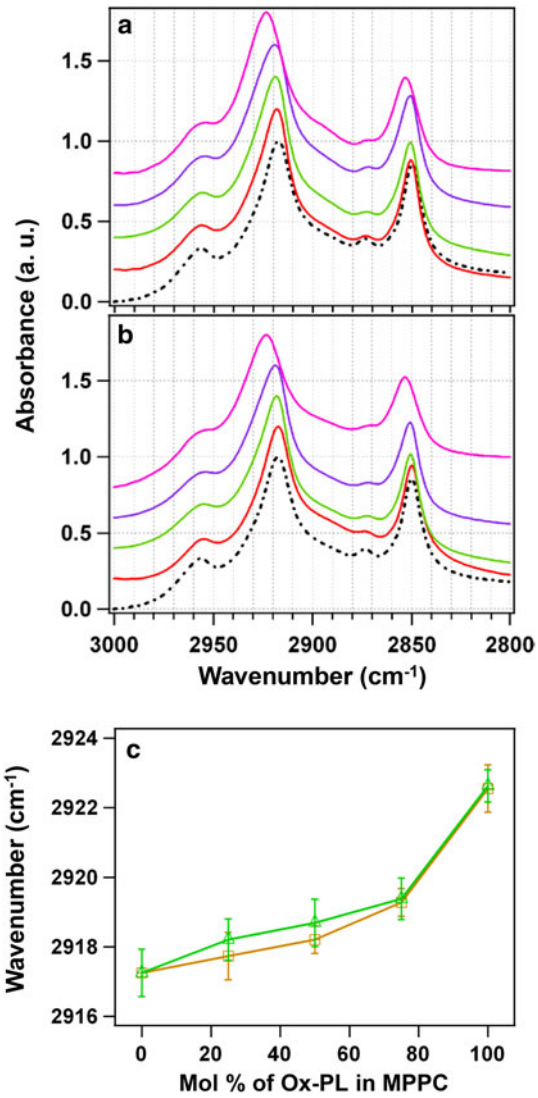

Fig. 5 ATR-FTIR spectra of lipid particles containing increasing concentrations of PGPC (**a**) and POVPC (**b**). Samples in D_2O-based buffer (100 µl of 5 mM) were deposited on the surface of crystal of the BioATRCell™ I, and the spectra were recorded at 25 °C. For each sample 526 interferograms were accumulated at a spectral resolution of 2 cm^{-1}. Spectra were baseline-corrected by rubberband corrections (OPUS software), and baseline-corrected spectra were used to determine the peak positions and plotted as a function of tOx-PL concentrations (**c**). In (**a**) and (**b**) *dotted line*, *red line*, *green line*, *purple line* and *pink line* represent 100 % MPPC, 75 % MPPC + 25 % tOx-PL, 50 % MPPC + 50 % tOx-PL, 25 % MPPC + 75 % tOx-PL and 100 % tOx-PL, respectively. *Triangle* and *square* represent PGPC and POVPC, respectively (Color figure online)

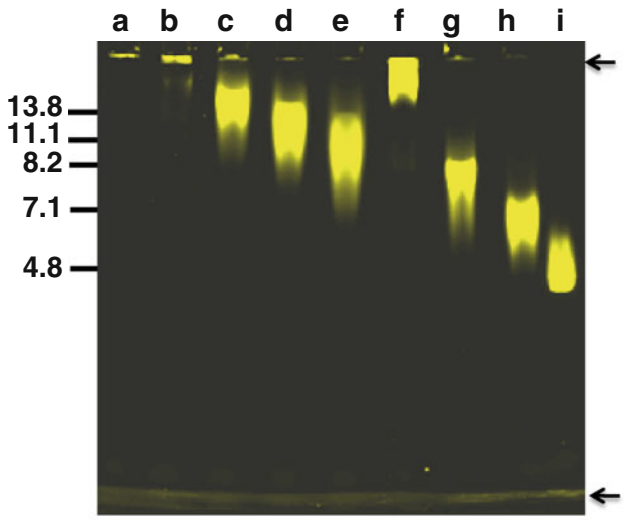

Fig. 6 Representative gel image showing native PAGE analysis of lipid particles. An aqueous suspension of 0.1 mol% NBD-DMPG (used as a fluorescent tracer)-labeled lipid particles containing increasing concentrations of tOx-PL were separated on 4–20 % gradient Tris–glycine gel. After electrophoresis, gels were directly imaged using a Typhoon Trio™ Variable Mode Imager with excitation wavelength of 488 nm (blue laser) and emission wavelength of 520 nm bandpass 40 filters. *Upper* and *lower arrows* point to the bottom of loading wells and the dye front, respectively. (*a*) 100 % MPPC, (*b*) 75 % MPPC + 25 % POVPC, (*c*) 50 % MPPC + 50 % POVPC, (*d*) 25 % MPPC + 75 % POVPC, (*e*) 100 % POVPC, (*f*) 75 % MPPC + 25 % PGPC, (*g*) 50 % MPPC + 50 % PGPC, (*h*) 25 % MPPC + 75 % PGPC, (*i*) 100 % PGPC

2008; Khandelia and Mouritsen 2009). Recently, a difference in the affinity of apolipoprotein-mimetic peptides toward closely related tOx-PLs has been reported (Epand et al. 2009). However, the molecular mechanism of this is not clear yet. In this report we have compared the properties of lipid particles containing POVPC and PGPC. The results suggest that the presence of POVPC and PGPC in the lipid particles leads to perturbation of normal packing of the lipids and causes changes in the acyl chain order (hydrocarbon region) as well as surface electrostatic properties of the bilayer. Lipid particles containing PGPC showed higher surface potential than lipid particles containing POVPC (Figs. 3, 7). It is known that a truncated *sn*-2 acyl chain bearing a polar functional group in Ox-PLs adopts a different conformation due to which the polar functional group of the truncated *sn*-2 acyl chain no longer remains buried within the hydrophobic interior, reverses its orientation and reaches the lipid–water interface (Beranova et al. 2010; Greenberg et al. 2008; Khandelia and Mouritsen 2009). The two tOx-PLs used in this study have oxidized acyl chains of the same length at the *sn*-2 position and differ only in the polarity of the terminal functional group. The results indicate that the *sn*-2 chain of tOx-PL reorients and protrudes into the aqueous surface of lipid particles, thereby disturbing the normal packing of acyl chains in the lipid bilayer.

More surface potential at the surface of lipid particles containing PGPC compared with POVPC-containing particles suggests that the ω-carboxyl group on the fatty acyl chain at the *sn*-2 position in PGPC is deprotonated and imparted a negative charge to the surface of the lipid

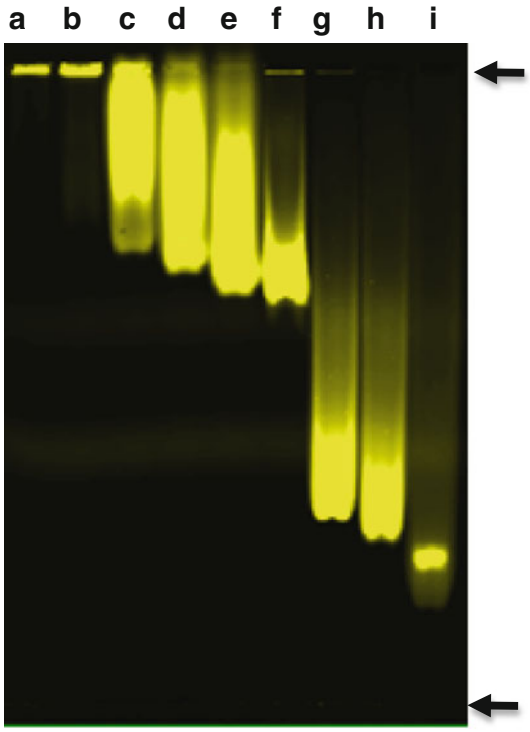

Fig. 7 Representative gel image showing agarose gel electrophoretic analysis of lipid particles. An aqueous suspension of 0.1 mol% NBD-DMPG (used as a fluorescent tracer)-labeled particles were separated on 0.5 % agarose gel (10 cm). After electrophoresis, gels were directly imaged using a Typhoon Trio^{TM} Variable Mode Imager using excitation wavelength of 488 nm (blue laser) and emission wavelength of 520 nm bandpass 40 filters. *Upper* and *lower arrows* in the gel point the bottom of loading wells and the dye front, respectively. (*a*) 100 % MPPC, (*b*) 75 % MPPC + 25 % POVPC, (*c*) 50 % MPPC + 50 % POVPC, (*d*) 25 % MPPC + 75 % POVPC, (*e*) 100 % POVPC, (*f*) 75 % MPPC + 25 % PGPC, (*g*) 50 % MPPC + 50 % PGPC, (*h*) 25 % MPPC + 75 % PGPC, (*i*) 100 % PGPC

particles, while the ω-aldehydic group remained uncharged at the surface. Taken together, our results suggest that when the size remains the same, the chemistry of the polar functional groups of the oxidized fatty acyl chain present in tOx-PLs dramatically modulates the physicochemical properties of lipid particles containing them.

The biological effects of Ox-PLs are mediated by the binding of Ox-PLs to receptor proteins expressed in various cell types (Kunjathoor et al. 2002; Podrez et al. 2002). However, it is realized that specific receptor binding is not the only way by which Ox-PLs exert their biological effects. Unique changes in the physicochemical properties due to the presence of these Ox-PLs in the membrane bilayer can modulate the membrane properties and play important role in various biological processes like cell adhesion, shape change, flattening, phagocytosis, microparticle shedding and chemotaxis (Huber et al. 2002, 2007; Kar et al. 2008; Beranova et al. 2010; Smith et al. 2009; Lis et al. 2011).

Thus, this result together with our previous report (Kar et al. 2011) provides direct evidence that because of their unique chemical compositions, Ox-PL species alter the physicochemical properties of lipid particles containing them in a typical way, which may be responsible for their considerably different biological effects.

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References

Bensikaddour H, Snoussi K, Lins L, Van Bambeke F, Tulkens PM, Brasseur R, Goormaghtigh E, Mingeot-Leclercq MP (2008) Interactions of ciprofloxacin with DPPC and DPPG: fluorescence anisotropy, ATR-FTIR and ^{31}P NMR spectroscopies and conformational analysis. Biochim Biophys Acta 1778:2535–2543

Beranova L, Cwiklik L, Jurkiewicz P, Hof M, Jungwirth P (2010) Oxidation changes physical properties of phospholipid bilayers: fluorescence spectroscopy and molecular simulations. Langmuir 26:6140–6144

Bochkov V, Oskolkova O, Birukov K, Levonen A, Binder C, Stöckl J (2010) Generation and biological activities of oxidized phospholipids. Antioxid Redox Signal 12:1009–1059

Catalá A (2009) Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. Chem Phys Lipids 157:1–11

Davis PJ, Keough KM (1985) Chain arrangements in the gel state and the transition temperatures of phosphatidylcholines. Biophys J 48:915–918

Deigner HP, Hermetter A (2008) Oxidized phospholipids: emerging lipid mediators in pathophysiology. Curr Opin Lipidol 19:289–294

Epand RF, Mishra VK, Palgunachari MN, Anantharamaiah GM, Epand RM (2009) Antiinflammatory peptides grab on to the whiskers of atherogenic oxidized lipids. Biochim Biophys Acta 1788:1967–1975

Fruhwirth GO, Loidl A, Hermetter A (2007) Oxidized phospholipids: from molecular properties to disease. Biochim Biophys Acta 1772:718–736

Fu P, Birukov KG (2009) Oxidized phospholipids in control of inflammation and endothelial barrier. Transl Res 153:166–176

Goormaghtigh E, Raussens V, Ruysschaert JM (1999) Attenuated total reflection infrared spectroscopy of proteins and lipids in biological membranes. Biochim Biophys Acta 142:105–185

Greenberg ME, Li XM, Gugiu BG, Gu X, Qin J, Salomon RG, Hazen SL (2008) The lipid whisker model of the structure of oxidized cell membranes. J Biol Chem 283:2385–2396

Huang C, Mason JT (1986) Structure and properties of mixed-chain phospholipid assemblies. Biochim Biophys Acta 864:423–470

Huber J, Vales A, Mitulovic G, Blumer M, Schmid R, Witztum JL, Binder BR, Leitinger N (2002) Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte–endothelial interactions. Arterioscler Thromb Vasc Biol 22:101–107

Huber LC, Jungel A, Distler JH, Moritz F, Gay RE, Michel BA, Pisetsky DS, Gay S, Distler O (2007) The role of membrane lipids in the induction of macrophage apoptosis by microparticles. Apoptosis 12:363–374

Johnstone SR, Ross J, Rizzo MJ, Straub AC, Lampe PD, Leitinger N, Isakson BE (2009) Oxidized phospholipid species promote

in vivo differential cx43 phosphorylation and vascular smooth muscle cell proliferation. Am J Pathol 175:916–924

Kar NS, Ashraf MZ, Valiyaveettil M, Podrez EA (2008) Mapping and characterization of the binding site for specific oxidized phospholipids and oxidized low density lipoprotein of scavenger receptor CD36. J Biol Chem 283:8765–8771

Kar S, Tillu VA, Meena SC, Pande AH (2011) Closely related oxidized phospholipids differentially modulate the physicochemical properties of lipid particles. Chem Phys Lipids 164:54–61

Kar S, Patel MA, Tripathy RK, Bajaj P, Suvarnakar UV, Pande AH (2012) Oxidized phospholipid content destabilizes the structure of reconstituted high density lipoprotein particles and changes their function. Biochim Biophys Acta 1821:1200–1210

Khandelia H, Mouritsen OG (2009) Lipid gymnastics: evidence of complete acyl chain reversal in oxidized phospholipids from molecular simulations. Biophys J 96:2734–2743

Kubica K, Langner M, Gabrielska J (2003) The dependence of fluorescein-PE fluorescence intensity on lipid bilayer state. Evaluating the interaction between the probe and lipid molecules. Cell Mol Biol Lett 8:943–954

Kunjathoor VV, Febbraio M, Podrez EA, Moore KJ, Andersson L, Koehn S, Rhee JS, Silverstein R, Hoff HF, Freeman MW (2002) Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. J Biol Chem 277:49982–49988

Leitinger N, Tyner TR, Oslund L, Rizza C, Subbanagounder G, Lee H, Shih PT, Mackman N, Tigyi G, Territo MC, Berliner JA, Vora DK (1999) Structurally similar oxidized phospholipids differentially regulate endothelial binding of monocytes and neutrophils. Proc Natl Acad Sci USA 96:12010–12015

Levitan I, Volkov S, Subbaiah PV (2010) Oxidized LDL: diversity, patterns of recognition, and pathophysiology. Antioxid Redox Signal 13:39–75

Lis M, Wizert A, Przybylo M, Langner M, Swiatek J, Jungwirth P, Cwiklik L (2011) The effect of lipid oxidation on the water permeability of phospholipids bilayers. Phys Chem Chem Phys 13:17555–17563

Mattila JP, Sabatini K, Kinnunen PK (2008) Interaction of cytochrome *c* with 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine: evidence for acyl chain reversal. Langmuir 24:4157–4160

Miller YI, Choi SH, Wiesner P, Fang L, Harkewicz R, Hartvigsen K, Boullier A, Gonen A, Diehl CJ, Que X, Montano E, Shaw PX, Tsimikas S, Binder CJ, Witztum JL (2011) Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. Circ Res 108:235–248

Nagle JF (1980) Theory of the main lipid bilayer phase transition. Annu Rev Phys Chem 31:157–196

Pande AH, Tillu VA (2011) Membrane lipid composition differentially modulates the function of human plasma platelet activating factor-acetylhydrolase. Biochim Biophys Acta 1811:46–56

Pande AH, Tripathy RK (2009) Preferential binding of apolipoprotein E derived peptides with oxidized phospholipid. Biochem Biophys Res Commun 380:71–75

Pande AH, Qin S, Tatulian SA (2005) Membrane fluidity is a key modulator of membrane binding, insertion, and activity of 5-lipoxygenase. Biophys J 88:4084–4094

Pande AH, Tripathy RK, Nankar SA (2009) Membrane surface charge modulates lipoprotein complex forming capability of peptides derived from the C-terminal domain of apolipoprotein E. Biochim Biophys Acta 1788:1366–1376

Pande AH, Kar S, Tripathy RK (2010) Oxidatively modified fatty acyl chain determines physicochemical properties of aggregates of oxidized phospholipids. Biochim Biophys Acta 1798:442–452

Parasassi T, De Stasio G, Ravagnan G, Rusch RM, Gratton E (1991) Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence. Biophys J 60:179–189

Parasassi T, Gratton E, Yu WM, Wilson P, Levi M (1997) Two-photon fluorescence microscopy of Laurdan generalized polarization domains in model and natural membranes. Biophys J 72:2413–2429

Podrez EA, Poliakov E, Shen Z, Zhang R, Deng Y, Sun M, Finton PJ, Shan L, Gugiu B, Fox PL, Hoff HF, Salomon RG, Hazen SL (2002) Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36. J Biol Chem 277:38503–38516

Sanchez SA, Tricerri MA, Ossato G, Gratton E (2010) Lipid packing determines protein–membrane interactions: challenges for apolipoprotein A-I and high density lipoproteins. Biochim Biophys Acta 1798:1399–1408

Smith HL, Howland MC, Szmodis AW, Li Q, Daemen LL, Parikh AN, Majewski J (2009) Early stages of oxidative stress–induced membrane permeabilization: a neutron reflectometry study. J Am Chem Soc 131:3631–3638

Soucaille P, Pratts M, Tocanne J, Teissie J (1988) Use of a fluorescein derivative of phosphatidylethanolamine as a pH probe at water/lipid interfaces. Biochim Biophys Acta 939:289–294

Sparks DL, Phillips MC (1992) Quantitative measurement of lipoprotein surface charge by agarose gel electrophoresis. J Lipid Res 33:123–130

Subbanagounder G, Leitinger N, Schwenke DC, Wong JW, Lee H, Rizza C, Watson AD, Faull KF, Fogelman AM, Berliner JA (2000) Determinants of bioactivity of oxidized phospholipids. Specific oxidized fatty acyl groups at the *sn*-2 position. Arterioscler Thromb Vasc Biol 20:2248–2254

Tamm LK, Tatulian SA (1997) Infrared spectroscopy of proteins and peptides in lipid bilayers. Q Rev Biophys 30:365–429

Tatulian SA (2003) Attenuated total reflection Fourier transform infrared spectroscopy: a method of choice for studying membrane proteins and lipids. Biochemistry 42:11898–11907

Taylor KMG, Morris RM (1995) Thermal analysis of phase transition behaviour in liposomes. Thermochim Acta 248:289–301