

MODELLING OF MOLECULAR MECHANISMS
OF MEMBRANE DOMAIN FORMATION DURING
THE OXIDATIVE STRESS: EFFECT OF PALMITOYL-
OXOVALEROYL-PHOSPHATIDYLCHOLINE

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Abstract

The study is focused on the effect of the oxidized lipid 1-palmitoyl-2-(5'-oxo-valeroyl)-*sn*-glycero-3-phosphocholine (POVPC) on the physicochemical properties of model membranes using two biophysical experimental methods: fluorescence video-microscopy and fluorescence spectroscopy. Two types of ternary mixtures made of sphingomyelin (SM), cholesterol (Chol) and respectively, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (PDPC) were used as a model system mimicking the lipid composition of specialized cellular membrane domains, called rafts. The monounsaturated POPC and polyunsaturated (PDPC) permit revealing the effects of the degree of unsaturation at *sn*-2 position in the phosphatidylcholine (PC) species. Fluorescence microscopy showed a difference in the effect of POVPC on raft-like domain formation in POPC/SM/Chol and PDPC/SM/Chol mixtures. In the PDPC mixtures, the oxidized lipid increased the temperature of micron-scale L_o (liquid-ordered)/L_d (liquid-disordered) phase separation whereas in the POPC-containing ones,

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POVPC abolishes the phase separation. The results from fluorescence spectroscopy, giving information about the nano-scale L_o/L_d phase separation, demonstrate that POVPC promotes the raft-like domain formation in a stronger dose-dependent way in monounsaturated lipid matrix compared to the polyunsaturated one. Fluctuation spectroscopy revealed the strong softening of POPC bilayers upon addition of only 5 mol% of POVPC. We ascertained the fact that the oxidized lipid, POVPC, induced larger structural changes in the membrane organization of monounsaturated lipid matrix. Thus, the results describe a structurally protective role of docosahexaenoic acid against the presence of the oxidized lipid.

Key words: oxidized phospholipids, docosahexaenoic fatty acid, rafts

Introduction. Cellular damage due to lipid oxidation is strongly associated with basic physiological processes such as cell signalling and apoptosis and a number of pathological processes. The molecular level influence of oxidized phospholipids (OxPLs) on the structure and function of cell membranes is largely elusive. Therefore, it is of high importance to understand the structure and functions of lipid membranes in biological systems by using artificial membranes which permits studying possible alterations in the lipid bilayer properties. Cell membranes are heterogeneous structures in lateral and transversal direction. They consist of various raft-like and non-raft domains with distinct compositions and properties. Lipid rafts are usually defined as small and highly dynamic plasma membrane domains in liquid-ordered (L_o) phase, that are enriched in saturated phospholipids, SM and Chol within a sea of unsaturated lipids in liquid-disordered (L_d) phase. Physicochemical properties of the plasma membrane have been shown to play an essential role in cellular functionality. Among those properties, the lipid packing is of high importance because it spatially regulates raft formation. It is known that micro- and nano-sized domains are formed in the plasma membranes during the cell signalling and trafficking events [1]. POVPC, identified as a critical marker of atherosclerotic progression [2], is among the most physiologically active OxPLs, product from the oxidation of the unsaturated glycerophospholipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC). It was shown that POVPC binds to human macrophages via the platelet-activating factor receptor (PAF-R), but not all of the PAF-R stimulated genes are affected [3]. This suggests that POVPC regulatory function is also realized by alteration of physicochemical properties of cellular membranes. To establish the capacity of the oxidized lipid to form raft-like domains, we prepared complex artificial membranes made of PC/POVPC/SM/Chol mixtures to mimic the cell plasma membranes. We compared lipid mixtures differing only in fatty acid at *sn*-2 position of PC species: monounsaturated oleic acid (OA) in POPC and polyunsaturated docosahexaenoic acid (DHA) in PDPC. OA was chosen because it represents the most common unsaturated FA. DHA is the longest (22 carbons) and most unsaturated (6 double bonds) FA commonly found in biological systems [4]. This polyunsaturated fatty

acid (PUFA) belongs to the family of Ω -3 fatty acids. We demonstrated a difference in the mechanism of action of the bioactive compound POVPC on POPC- and PDPC-containing membranes and the protective role of DHA against the structural membrane changes induced by oxidized lipids.

Materials and methods. Reagents. The lipids used in our experiments were 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (PDPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-(5'-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC), egg-yolk sphingomyelin (SM), cholesterol (Chol) and fluorescent lipid analogue L- α -phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhod-PE). All these lipids were purchased from Avanti Polar Lipids. 2,2,6,6-Tetramethylpiperidine 1-oxyl (TEMPO) and fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were bought from Sigma Aldrich.

Preparation of Giant Unilamellar Vesicles (GUVs) and video microscopy. GUVs were prepared by using an electroformation method (AC field, 400 mV, 10 Hz) developed by ANGELOVA and DIMITROV [5]. GUVs were observed using a Zeiss Axiovert 135 microscope. Vesicles were followed in phase contrast and in fluorescence by Zeiss filter set 15 (Ex/Em = 546/590 nm). Fluorescence microscopy method is able to visualize the micron-scale phase separation and to determine the L_o/L_d phase separation temperature. The fluorescent lipid marker egg yolk Rhodamine PE is known to partition predominantly in L_d phase thus visualized as bright membrane regions under fluorescence [6] and L_o phase appearing as dark round-shaped domains within the vesicle membrane. GUV's formation is performed at temperatures above the temperature of L_o/L_d miscibility. The temperature of micron-scale miscibility transition was assessed as the temperature of the first domain appearance and disappearance upon cooling and after reheating, respectively. The average of these two values could determine the micron-scale miscibility transition temperature (T_m). The vesicles were studied in the temperature range from 65 °C to 4 °C at rate 0.1 °C/min.

Preparation of Large Unilamellar Vesicles (LUVs). Large unilamellar vesicles (LUVs) were prepared by extrusion method [7] for fluorescence spectroscopy measurements of DPH. The capacity of lipid mixtures to form nano-scale raft-like domains was probed by DPH-TEMPO quenching method. DPH molecules partition into both coexisting phases (L_d and L_o) [8] whereas TEMPO as a water-soluble molecule diffuses preferentially into the L_d phase [9]. Thus, TEMPO quenches effectively the fluorescence from DPH in the disordered domains and weakly the DPH fluorescence originating from the ordered domains. The remaining DPH fluorescence in the presence of TEMPO (Q) was defined as:

$$(1) \quad Q = F/F_0,$$

where F and F_0 are the fluorescence intensities of DPH in the presence and absence of TEMPO, respectively [10]. The quenching of DPH fluorescence by TEMPO was

measured at 37 °C with a SynergyTM2 (BioTek) spectrofluorimeter. Fluorescence was recorded at 360/40 nm excitation and 460/40 nm emission. Samples were run in triplicate.

Results. The physicochemical properties and structural organization of model membranes containing the oxidized lipid POVPC were investigated. In our experiments, GUVs and LUVs were prepared from two types of mixtures used as controls: POPC/SM/Chol and PDPC/SM/Chol (34:33:33 mol/mol). Certain amounts of PC species were replaced by POVPC in PC/SM/Chol control mixtures in order to mimic the oxidation process in the membrane and the generation of the oxidized lipids. The following three POVPC concentrations were examined: 2.5, 9 and 17 mol% at the two types of optic methods, microscopic and spectroscopic ones.

1. Visualization of raft-like domain formation induced by POVPC in model membranes (GUVs) by fluorescence microscopy. The control GUVs made of POPC/SM/Chol 34:33:33 mol/mol ternary mixture were electroformed at 45 °C. In this mixture, the phase separation was observed in the temperature range from 45 °C to 25 °C as the L_o/L_d micron-scale miscibility transition temperature (T_m) has been determined in our previous study to be 30.3 ± 2.6 °C [11]. POPC-containing vesicles exhibited homogeneous appearance from 45 °C to 33 °C (data not shown) [11]. At 32 °C first bright domains were observed (Fig. 1a). The visualization of the phase separation was achieved due to the preferential partition of the fluorescent probe egg yolk Rhod-PE into the loosely packed L_d phase which appeared as the bright phase in the vesicle membrane [12]. The exclusion of the dye from the more ordered L_o phase made it look as dark regions when observed by fluorescence. Thus, bright domains in L_d phase appeared on continuous dark background in L_o phase in Fig. 1a. The addition of only 2.5 mol% POVPC to the control POPC/SM/Chol mixture shows a lack of L_o/L_d micron-scale phase separation (homogeneous vesicles) during the whole studied temperature range, from 45 °C to 4 °C (Fig. 1b). The control GUVs composed of PDPC/SM/Chol 34:33:33 ternary mixture were also electroformed at 45 °C similar to POPC ternary mixtures. The vesicles were then heated to 65 °C and the fluorescence observation was observed from 65 °C to 4 °C. The L_o/L_d micron-scale miscibility transition temperature of this mixture has been determined in our previous study to be 60.2 ± 1.9 °C [11]. Large one or two domains at 37 °C are regularly observed as the fraction of L_o domains covers more than half of the surface area of the vesicle (Fig. 1c). The addition of 2.5 mol% POVPC to the control mixture increases the fraction of L_o domains by decreasing the L_d fraction, as it is shown in Fig. 1d at the same temperature.

Fluorescence microscopy data showed that the oxidized lipid alters the raft-like domain formation in both types of PC mixtures. POVPC in POPC-containing mixtures leads to disappearance of the micron-scale L_d domains whereas in PDPC-containing ones the oxidized lipid only decreases their fraction.

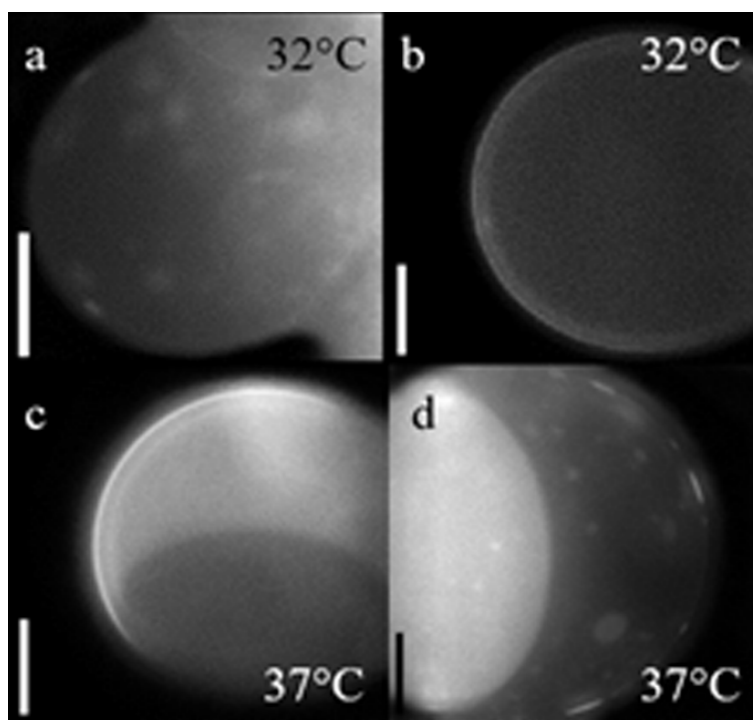


Fig. 1. Effect of POVPC on the raft-like domain formation in POPC/SM/Chol 34:33:33 mol/mol (first row) and PDPC/SM/Chol (second row). Micron-scale L_d domains on L_o background are observed in the control POPC/SM/Chol mixture at 32 °C (a). Disappearance of the L_d domains at 32 °C in POPC/POVPC/SM/Chol mixture (31.5:2.5:33:33) (b). Large L_o domains on L_d background are observed in PDPC/SM/Chol at 37 °C (c). The addition of 2.5 mol% POVPC decreases the fraction of the bright L_d domain and, respectively, increases the dark L_o one at the same temperature (d). Scale bar: 10 μ m

2. Effect of POVPC on the nano-scale raft domain formation studied by DPH-TEMPO fluorescence spectroscopy. To assess the effect of POVPC on the nano-scale raft domain formation, the Q value of liposome is calculated in monounsaturated and polyunsaturated lipid matrices (Fig. 2a). The dashed and dotted lines describe Q values for single-component PC and binary SM/Chol 1:1 vesicles, respectively. The higher Q value indicates the lower DPH quenching by TEMPO. The single-component PC vesicles (model of “pure” L_d phase) exhibit lower Q values (50–60%) compared to the binary SM/Chol 1:1 mixture (model of L_o phase) (93%) which denotes higher DPH quenching for L_d phases and much lower one for the L_o phase. The observed differences in quenching for the L_d and L_o phases could be assigned to lower lipid order in the L_d phases and higher one in the L_o phase. The Q value for L_d polyunsaturated matrix is higher than in monounsaturated one which stands for lower quenching of DPH

molecules. Unexpectedly, the PDPC membranes show a higher lipid order than POPC ones, although the fatty acid at *sn*-2 position of the molecule is DHA, which contains six double bonds and should form phases with lower molecular order. This result does not contradict to HO et al. [13] statement that there is a clear correlation between the hydration of acyl chains and their order, as opposed to the correlation between acyl chain order and polar head hydration. This can be explained by the fact that *sn*-1 chains show decreased order at the chain end (bilayer centre) but increased order in the vicinity of the lipid headgroups in the presence of DHA at *sn*-2 position [14]. PDPC ternary vesicles show higher degree of lipid packing in the membrane similar to the single-component ones. To identify the capacity of lipids to form raft-like domains, we calculated ΔQ defined as the difference between DPH quenching (Q) for ternary lipid mixtures and Q for pure PC liposomes (Q_{PC}) divided by $(Q_{SM/Chol(1:1)} - Q_{PC})$ (Fig. 2b):

$$(2) \quad \Delta Q_{\text{raft mixtures}}(\%) = ((Q_{PC/SM/Chol} - Q_{PC}) / (Q_{SM/Chol(1:1)} - Q_{PC})) * 100$$

The rationale to calculate this value comes from the fact that pure PC (L_d phase only) vesicles exhibit different Q values. This originates from the different structural characteristics of each PC containing bilayer such as molecular order parameter as well as bilayer thickness determining different membrane permeability and transversal diffusion of water-soluble molecule TEMPO. These physicochemical properties control the yield of DPH quenching by TEMPO beyond the raft domain formation. By calculating ΔQ , these intrinsic PC structural specificities

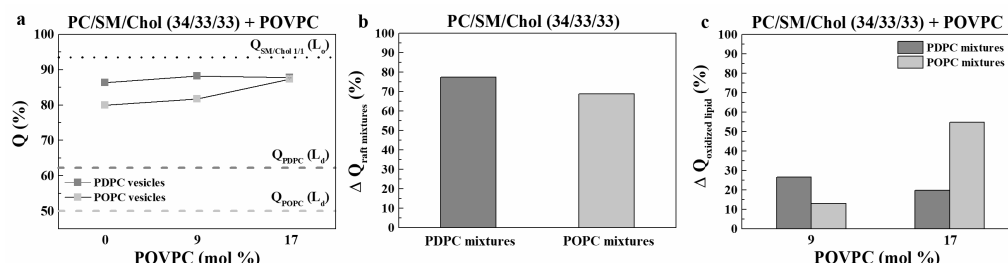


Fig. 2. DPH fluorescence quenching by TEMPO: remaining DPH fluorescence in the presence of TEMPO (Q): (a) PC (PDPC or POPC)/SM/Chol 34:33:33 mol/mol mixtures with and without POVPC. The dotted line indicates the Q value in raft-like L_o membranes (SM/Chol 1:1) and the dashed lines – in pure L_d membranes (PDPC and POPC). All measurements were performed at 37 °C. Each point corresponds to the mean value of three experiments. Error bars were too small to be displayed. (b) $\Delta Q_{\text{raft mixtures}}$ as a function of control lipid mixtures. ΔQ is defined as the difference between DPH quenching (Q) for ternary lipid mixtures and 100% PC liposomes (Q_{PC}) divided by $(Q_{SM/Chol(1:1)} - Q_{PC})$: $\Delta Q_{\text{raft mixtures}}(\%) = ((Q_{PC/SM/Chol} - Q_{PC}) / (Q_{SM/Chol(1:1)} - Q_{PC})) * 100$. (c) $\Delta Q_{\text{oxidized lipid}}$ as a function of POVPC concentration in lipid mixtures. ΔQ denotes the difference between Q of raft mixtures with and without POVPC, divided by $(Q_{SM/Chol(1:1)} - Q_{PC/SM/Chol})$: $\Delta Q_{\text{oxidized lipid}}(\%) = ((Q_{PC/SM/Chol+POVPC} - Q_{PC/SM/Chol}) / (Q_{SM/Chol(1:1)} - Q_{PC/SM/Chol})) * 100$. ΔQ is calculated from the average Q values of 3 samples

are “subtracted” and only the capacity of different PC to form raft-like domains in the ternary mixtures is assessed. Making the assumption that the total fraction of L_o -phase domains in the lipid membrane is proportional to the unquenched DPH fluorescence, the term “capacity to form nano-scale raft-like domains” could be introduced. So, the higher the unquenched DPH fluorescence is, the higher the “capacity to form raft-like domains” would be. In POPC/SM/Chol 34:33:33 mol/mol mixture the fraction of L_o domains is 11% lower compared to PDPC-containing ternary one at physiological temperature. The addition of 9 and 17 mol% POVPC to the ternary mixtures increases the lipid order parameter into both PC matrices. POVPC at 9 mol% increases the Q value in both types of PC ternary mixtures as to a greater extent at PDPC-containing mixture, whereas 17 mol% POVPC induces greater changes in Q for POPC ternary mixture compared to PDPC one (Fig. 2c). It can be clearly seen that POVPC promotes the raft-like domain formation in stronger dose-dependent way in monounsaturated lipid matrix unlike polyunsaturated one.

Discussion. The results obtained by the two independent methods suggest a different mechanism of action of the oxidized lipid in monounsaturated and polyunsaturated ternary mixtures. Fluorescence microscopy of GUVs and fluorescence spectroscopy of LUVs allowed us to investigate directly the impact of oxidized phospholipid POVPC on raft-like domain organization in PC (POPC or PDPC)/SM/Chol mixtures. We demonstrated the ability of POVPC to control the lateral organization of membrane lipids in POPC mixtures (domain pattern, lipid order and raft fraction). This effect was much lower in PDPC mixtures at the same lipid composition. Such difference between two types of lipid mixtures on affecting membrane lateral organization may be attributed to the distinct fatty acid at *sn*-2 position of PC. We suggest the following putative mechanism of action of POVPC on the membrane lateral organization. In ternary control POPC/SM/Chol 34:33:33 mol/mol mixtures, the vesicles exhibited homogeneous appearance at 37 °C by fluorescence microscopy. However, our previous ESR studies [11], as well as here presented DPH-TEMPO method based on direct contact quenching of the two molecules (< 12 Å) [9,10], have revealed L_o/L_d phase separation in the nanometer scale at this temperature. As the temperature decreases to 32 °C, homogeneous vesicles became heterogeneous ones, where small bright micron-scale L_d domains appeared on a dark L_o background. The formation of such domains at temperatures so close to the physiological one suggests also the existence of nanometer-scale domains at this temperature. The addition of very small amount of POVPC removes L_o/L_d phase separation by its direct partition into the L_o phase and interaction with Chol. POVPC is a truncated oxidized lipid, having an inverted conical shape. KHANDELIA et al. [15] proposed that a conical molecule (e.g. Chol) and an inverted conical molecule (truncated lipid) should seek each other and pair-up in order to pack together into planar membranes composed of otherwise predominantly cylindrical phospholipids. The mechanism

of action of POVPC in PDPC-containing mixtures is opposite. Relatively large micron-scale L_d domains in PDPC/SM/Chol mixtures were observed at a physiological temperature due to the low affinity of Chol to PUFAs standing for much higher line tension of the domains compared to POPC ternary mixture [16]. The low affinity of Chol for PDPC in L_d phase can explain its segregation into the L_o phase allowing more SM molecules to segregate along with Chol [11]. The presence of POVPC increased the fraction of L_o domains which led to further increase in their line tension.

Oxidized lipids are indicators of both physiological (inflammation, proliferation and apoptosis) and pathological processes in the cell. In the last few years, scientists have become increasingly interested in the behaviour of amyloid peptide ($A\beta$) in Alzheimer's disease (AD) [17]. Many studies also focus on oxidized lipids due to their involvement in pathological conditions such as AD. Recently, some studies have shown that oxidized lipids are an important factor in AD because they can indirectly promote surface β -amyloid production and accumulation through lipid raft formation [18]. Other possible reason might be that oxidized lipids change directly lipid rafts composition, structure and functions [19]. The coalescence of "raft" nanodomains in microdomains is a tool for the cell signalling. Many of the signalling processes into the neurons are affected at different levels of AD. Such disorders in the signalling pathway may be associated with the effect of oxidized lipids on raft formation.

Conclusions. In conclusion, we have shown that the oxidized lipid, POVPC, impacts physicochemical behaviour of lipid model membranes. It could be assumed that the effect of this bioactive compound on the membrane bilayer depends on the degree of unsaturation of the fatty acid in the PC molecule. The effect of POVPC is more pronounced in POPC-containing membranes (monounsaturated matrix) compared to PDPC one (polyunsaturated matrix). There has been considerable interest in the effect of DHA for human health lately. The research assessing DHA in relation to brain development and brain function has increased. So, we suggest that the DHA belonging to the Ω -3 family is able to compensate the alterations induced by the presence of oxidized lipids in the membranes. It demonstrates a model of structural protective role against the generation of oxidized lipids during the membrane related oxidation processes in the cell.

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