

Role of Aminophospholipids in the Formation of Lipid Rafts in Model Membranes

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Abstract The phase separation of aminophospholipids in glycerophospholipid matrix and the effect of cholesterol were studied by means of fluorescence microscopy of giant unilamellar vesicles (GUV). GUVs were composed of binary mixtures, egg yolk phosphatidylcholine (eggPC)/egg yolk phosphatidylethanolamine (eggPE) and egg yolk phosphatidylcholine (eggPC)/brain phosphatidylserine (brainPS), and ternary ones with both aminophospholipids (eggPC/eggPE/brainPS). Gel/liquid-disordered phase coexistence was detected in these mixtures, where aminophospholipids segregate in gel leaf-like domains. When cholesterol (CHOL) was added, the phase separation was shifted at lower temperatures. CHOL increases miscibility of aminophospholipids in PC matrix. Addition of PE and PS to the ternary mixtures (eggPC/eggSM/CHOL) induced liquid-ordered domain formation at higher temperatures. Based on these results, one can conclude that aminophospholipids promote the formation of L_o domains.

Keywords Phosphatidylethanolamine · Phosphatidylserine · Rafts · Liquid-ordered phase · Inner leaflet

Introduction

Aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), are important structural elements

building the inner cytoplasmic leaflet of the cell plasma membrane. While the choline-containing phospholipids, phosphatidylcholine (PC) and sphingomyelin (SM), form extracellular lipid leaflet, cholesterol (CHOL) is considered to be distributed equally between both leaflets in the most of cell types [1]. The lipids in plasma membranes do not mix ideally in the membrane bilayer and they are organized in specialized lipid domains. Membrane domains, called lipid rafts, differ in their lipid and protein contents from the bulk of the membrane [2, 3]. Although still controversial, rafts are thought to be involved in numerous cellular process, including cell signaling, trafficking, and bacterial infection [4–6]. Lipid rafts can be defined as sphingolipid and sterol-rich domains that exist in the liquid-ordered (L_o) state. These domains are thought to coexist with liquid-disordered (L_d)-state domains rich in lipids with unsaturated acyl chains [2, 7]. Bilayers in L_o -state form when cholesterol is mixed with a lipid characterized with a high gel (L_β)/ L_d melting temperature. Studies on model bilayers containing ternary mixtures of CHOL, SM and unsaturated PC demonstrated liquid/liquid immiscibility [8, 9]. Apparently, the formation of micron-scale liquid/liquid immiscibility is a specific feature of CHOL and phosphocholine-containing species, such as SM and saturated PC because such phase behavior was not observed for structurally-identical of SM species, ceramide and sphingosine [10, 11].

In the last few years the attention is focused on the hypothesis about the existence of lipid rafts in the inner leaflet of the cell plasma membrane. The main question on this subject is what the role of aminophospholipids on the formation of raft-like domains is. It was demonstrated that the strength of an interaction between PE and CHOL depends on the degree of saturation of the phospholipid [12, 13]. Increasing unsaturation in PE results in increased phase separation from SM/CHOL membranes [14]. Moreover, if fatty acids content is identical in phospholipids, miscibility of CHOL in

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phospholipid/CHOL mixtures decreases in the order: PC > PE > PS [15, 16]. It appears that the presence of either a net charge on the headgroup or interheadgroup hydrogen bonding acts to reduce cholesterol solubility. The presence of both factors has a reinforcing effect [16]. Obviously a crucial role, about the strength of interaction of phospholipids with CHOL and the formation of raft-like domains, plays the polar headgroup of the lipid. DSC experiments showed that the magnitude of the reduction in the phase transition temperature induced by CHOL addition is independent of the hydrocarbon chain length of the PS and PE bilayers [17, 18]. Unlike these aminophospholipids, in PC bilayers, CHOL increases or decreases the phase transition temperature in a chain length-dependent manner [19]. Finally, McElhaney's group [6] concluded that the effect of CHOL on the thermotropic phase behavior of the host phospholipid bilayer depends on the strength of the attractive interactions between the polar headgroups and the hydrocarbon chains of the phospholipid molecule, and not on the charge of the polar headgroups per se.

Bakht et al. [20] ascertained that even in the absence of high-T_m lipids, mixtures of CHOL with PE and PS showed a tendency to form ordered domains. Moreover, these authors found that in bilayers composed of ternary mixtures of palmitoyl-oleoyl glycerophospholipids (PE or PS), cholesterol, and high-T_m lipids, the thermal stability of ordered domains decreased in the following order: PE > PS > PC [20].

Plasma membrane cytoplasmic leaflet contains SM and CHOL not as high level as exoplasmic leaflet but enough to serve as a prerequisite for the formation of lipid rafts. That is why we focused our attention to clarify the capacity of each aminophospholipid (PE and PS) and combination of both, in the formation of micron-scale domains in L_o state. In this study we used as model membranes, giant unilamellar vesicles (GUVs), monitored by fluorescence microscopy, to visualize firstly micron-scale immiscibility of aminophospholipids in PC environment. As second step, we studied the effect of cholesterol on aminophospholipids immiscibility in mixtures composed of PC/aminophospholipids/CHOL. Aminophospholipids effect on the formation of micron-scale L_o domains was investigated as a third step.

Materials and Methods

Reagents Egg yolk L- α -phosphatidylcholine (eggPC), egg yolk L- α -phosphatidylethanolamine (eggPE), bovine brain L- α -phosphatidylserine (brainPS), egg yolk sphingomyelin (eggSM), cholesterol (CHOL). The distribution of fatty acids in eggPC consisted of 34 % C16:0, 2 % C16:1, 11 % C18:0, 32 % C18:1, 18 % C18:2 and 3 % C20:4; eggPE-17 % C16:0, 0.5 % C16:1, 24 % C18:0, 18 % C18:1, 14 % C18:2, 0.2 % C20:2, 0.3 % C20:3, 16 % C20:4, 4 % C22:6 and 5.2 %

unknown; brainPS- 42 % 18:0, 30 % 18:1, 2 % 20:4, 11 % 22:6, 15 % unknown; eggSM-84 % C16:0, 6 % C18:0, 2 % C20:0, 4 % C22:0 and 4 % C24:0 The used fluorescent lipid analogue was Acyl 12:0 NBD PC (1-acyl-2-{12-[(7-nitro-2-1, 3-benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphocholine. All lipids were obtained from Avanti Polar Lipids. Hepes buffer was purchased from Sigma-Aldrich.

Preparation of Giant Unilamellar Vesicles and Video Microscopy

The electroformation is widely used method to produce giant unilamellar vesicles (GUVs) [21]. These vesicles represent a model membrane system, which has similar size as living cell (10–100 μ m in diameter) [22]. Binary, ternary, quaternary and quinary mixtures of eggPC, eggPE, eggSM, brainPS and CHOL were prepared in the required lipid molar ratios using 70/10/20 v/v diethylether/methanol/chloroform solution. The total lipid concentration was 0.5 mg/ml. The method of GUV preparation includes: a spread of 1.2 μ l from the lipid mixture on the two parallel platinum wires (diameter 0.8 mm, distance between axes 3 mm) of the working chamber under a stream of nitrogen. Lipid films were dried under vacuum for at least 45 min. The working chamber is mounted on a Peltier microscope stage in which the rate of temperature change is 0.1 $^{\circ}$ C/min. Thermocouple is positioned at a distance of about 0.5 mm from the one of the electrode. Before hydration step with warm buffer (0.5 mM Hepes, pH 7.4, at 45 $^{\circ}$ C), the desired temperature is achieved by the thermostatic system. GUVs electroformation starts with application of 10 Hz alternating current (AC), 100 mV peak-to-peak (pp) at 45 $^{\circ}$ C. The applied voltage is gradually increased to 400 mV pp during 25 min. After about 3 h, at least 20 GUVs of diameters 30–90 μ m were available for observation. A coverslip was used to avoid buffer evaporation.

To avoid lipid oxidation during the fluorescence observation and the electroformation procedure, the following preventive measures were undertaken as it is recommended by Zhao et al. [23]. Low power illumination was applied to the sample by using of 50 W Hg arc lamp light, additional filter to decrease the power of UV rays, maximum closed aperture and low dye concentration (2 mol % C12 NBD PC). Such dye concentration, according to Bouvrais et al. [24], does not exhibit strong perturbing effect on the membrane properties but peroxide formation may be a potential problem at higher dye content and/or powerful illumination. A low 400 mV pp sine wave voltage was applied unlike other reports where ITO-glass electrodes were used and much higher voltages (1.4–10 V) were applied [25, 26]. Moreover, we accepted as experimental results the phase separation visualized immediately after opening of illumination and not those taken after long time of observation.

To visualize lipid phase separation, fluorescence microscopy was used. The vesicles were observed using a Zeiss

Axiovert 135 microscope, equipped with a 40× long working distance objective lens. The observations were recorded using Hamamatsu B/W chilled CCD camera connected to an image recording and processing system. Fluorescence experiments were carried out by using Zeiss filter set 16 (Ex/Em=485/>520 nm). Fatty acid labeled fluorescent dye C12 NBD PC (2 mol %) is excluded from the more ordered phase and preferentially partitions in liquid-disordered phase [27]. Thus, more ordered phases appeared as dark regions on the bright background.

The term “micron-scale miscibility transition temperature” was firstly described by Veatch and Keller [28]. This phenomenon corresponds to the temperature at which domain formation at micron-scale can be observed. Its assessment can be done by the observation of the appearance and disappearance of domains during a temperature cooling and subsequent heating. Transition temperature is defined as the average of these two points. Standard deviations correspond to the averaged data from 30 vesicles. Three experiments were systematically carried out for each mixture as at least 10 vesicles were probed per experiment.

It is noteworthy the value of the micron-scale miscibility transition temperature depends on the objective’s magnification.

Results and Discussion

L_{β}/L_d Immiscibility of Aminophospholipids in Glycerophospholipid Matrix

To determine micron-scale miscibility transition temperature of aminophospholipids in glycerophospholipid matrix and morphology of the observed domains in GUV membranes, the following binary lipid mixtures were studied: eggPC/eggPE (85/15 mol/mol), eggPC/brainPS (85/15) and ternary mixtures including the two aminophospholipids, eggPC/brainPS/eggPE (70/15/15). The molar percentage of aminophospholipids was chosen to mimic the amounts of these lipids in inner leaflet of the cell plasma membranes. The asymmetric distribution of aminophospholipids in the plasma membranes was not concerned in this study because the membranes of GUVs are symmetric. Herein, only the capacity of aminophospholipids to form domains has been evaluated. In the studied lipid mixtures vesicles remain with homogeneous appearance in wide temperature range from 37 °C to 15 °C (Fig. 1a). Dark leaf-like domains on bright background were observed for all mixtures when temperature was decreased (Fig. 1b–d). Because these domains did not grow by fusion and their shape was not round, all these characteristics set the pattern for gel (L_{β})/liquid (L_d) immiscibility in the lipid bilayer. It might be supposed that the dark leaf-like domains result from the self-aggregation of PE and PS molecules thus

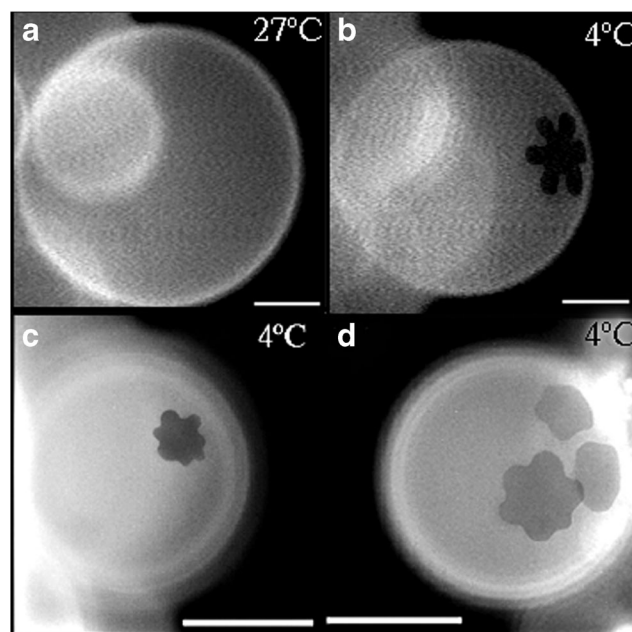


Fig. 1 Fluorescence micrographs of GUVs, composed of 85/15 eggPC/eggPE (a and b), eggPC/brainPS (c) and mixture including the two aminophospholipids—eggPC/brainPS/eggPE 70/15/15 (d). Homogeneous appearance in the temperature range from 37° to 15 °C was observed for 85/15 eggPC/eggPE mixture (a). Dark leaf-like domains on bright background were detected below 15 °C for all mixtures (b–d). Scale bar 20 μm

forming domains in gel phase. The temperature of L_{β} domain formation was determined according to the rules described in Veatch et al. [28]. Brief description of this procedure is also given in the section **Materials and Methods**. The L_{β}/L_d miscibility transition temperatures for these mixtures are summarized in Table 1. The lowest miscibility transition temperature was detected for eggPC/brainPS 85/15 (8.1 °C), followed by eggPC/eggPE 85/15 (10.4 °C) and eggPC/brainPS/eggPE 70:15:15 (13.9 °C). It is noteworthy that the shape of the dark leaf-like domains is the most forceful marked in 85/15 eggPC/eggPE mixture (Fig. 1b) compared to the other mixtures (Fig. 1c–d). Long, well formed petals were always observed in this 85/15 eggPC/eggPE mixture. As it is expected, the apparent fraction of the leaf-like domains is larger in 70/15/15 eggPC/brainPS/eggPE (Fig. 1d, Table 1) unlike others due to higher molar ratio of aminophospholipids in the mixture. However, the petals of these leaf-like domains are smaller unlike in eggPC/eggPE mixture (Fig. 1b and d). The presence of charged lipid (PS) appears to be a factor determining the domain morphology of aminophospholipids in glycerophospholipid matrix.

Effect of Cholesterol on Aminophospholipids Miscibility

The addition of cholesterol decreased the miscibility transition temperatures and apparent fraction of leaf-like domains (Table 1, Fig. 2). The greatest effect was observed for the

Table 1 Micron-scale miscibility transition temperature of the studied aminophospholipids-containing mixtures. Temperature error bars were estimated by observing the appearance and disappearance of domains respectively when temperature was decreased and increased. The

apparent fractions of dark phase and error bars correspond to an average over 10 GUVs for each point. Such fractions were measured with ImageJ [29] and are only apparent since only one side of the GUV surface was visible and no curvature correction was applied

Mixtures	Micron-scale miscibility transition temperature (T_m)	Apparent fraction of dark phase (L_β or L_o) at 4 °C
eggPC/eggPE 85/15 (L_β/L_d)	(10.4±2.1)°C	0.069±0.015
eggPC/brainPS 85/15 (L_β/L_d)	(8.1±2.6)°C	0.051±0.017
eggPC/brainPS/eggPE 70/15/15 (L_β/L_d)	(13.9±1.8)°C	0.236±0.041
eggPC/eggPE/CHOL 65/15/20 (L_β/L_d)	(4.3±1.4)°C	0.007±0.002
eggPC/brainPS/CHOL 65/15/20 (L_β/L_d)	(7.7±1.9)°C	0.050±0.011
eggPC/brainPS/eggPE/CHOL 50/15/15/20 (L_β/L_d)	(6.2±1.5)°C	0.022±0.017
eggPC/eggSM/CHOL 60/20/20 (L_o/L_d)	(20.3±1.4)°C	0.276±0.066
eggPC/eggPE/eggSM/CHOL 50/10/20/20 (L_o/L_d)	(25.1±1.8)°C	0.485±0.038
eggPC/brainPS/eggSM/CHOL 50/10/20/20 (L_o/L_d)	(22.8±1.7)°C	0.242±0.022
eggPC/brainPS/eggPE/eggSM/CHOL 40/10/10/20/20 (L_o/L_d)	(25.4±2.2)°C	0.554±0.049

quaternary mixture eggPC/brainPS/eggPE/CHOL. Intermediate effect was found for the eggPE-mixture and light effect for the brainPS-mixture. Apparently, the presence of eggPE

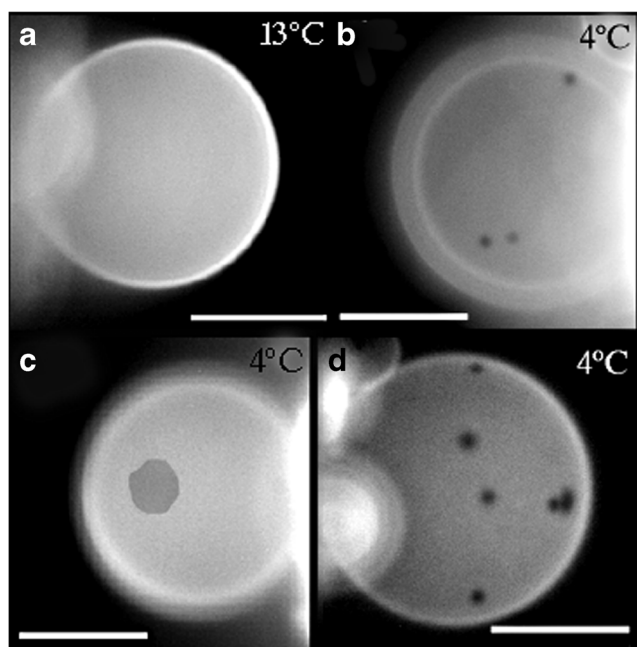


Fig. 2 Effect of cholesterol on aminophospholipids L_β/L_d immiscibility. GUVs composed of eggPC/eggPE/CHOL 65/15/20 (a and b), eggPC/brainPS/CHOL 65/15/20 (c) and eggPC/brainPS/eggPE/CHOL 50/15/15/20 (d) mixtures. The presence of cholesterol decreased the temperature of domain formation, changed larger domain morphology and size only when eggPE is present in the mixtures (b and d). Such effect was not observed for 65/15/20 eggPC/brainPS/CHOL mixture compared to the control, without CHOL (85/15 eggPC/brainPS). Scale bar 20 μ m

increased the miscibility of brainPS for the liquid-disordered phase in the quaternary mixture eggPC/brainPS/eggPE/CHOL because the cholesterol itself was not able to do it in the ternary one (eggPC/brainPS/CHOL). As it could be seen in Fig. 2b and c, larger gel domains have been still observed in brainPS-mixture. The disability of small dark domains to merge and yield larger ones represents a proof that these are domains in gel phase (Fig. 2).

Effect of Aminophospholipids, eggPE and brainPS, on L_o/L_d Micron-Scale Immiscibility

Control GUVs were composed of 60/20/20 eggPC/eggSM/CHOL mixture (Fig. 3a and b). Dark round-shaped L_o domains appeared at 20.3 °C (Table 1). For clarity, the visualization of L_o domains in all studied mixtures was always shown at slightly lower temperatures than the temperature of their formation. The domains in L_o phase grew in size by fusion which represents a characteristic property of the liquid/liquid immiscibility. Their domain size increased with temperature decrease (4 °C) (Fig. 3b). Addition of 10 mol% eggPE to the control mixture shifted the temperature of domain formation to higher temperatures (Table 1). Thus, the fraction of L_o domains was larger than that in the control mixture at 4 °C (Fig. 3d and b, Table 1). The presence of brainPS (10 mol%) in the quaternary mixtures eggPC/brainPS/eggSM/CHOL also shifted the L_o domain formation to higher temperatures but this effect is less pronounced compared to eggPE-mixture (Fig. 3e). However, the L_o fraction in brainPS-mixture was smaller compared to eggPE-one (Fig. 3d and f, Table 1). The presence of the two

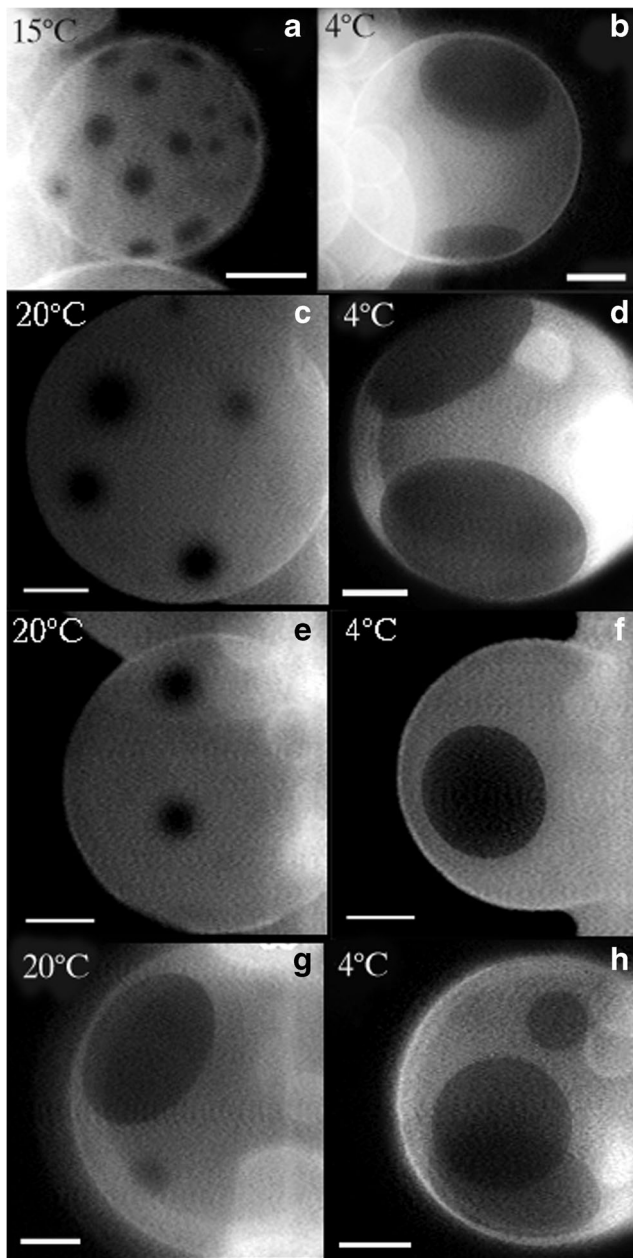


Fig. 3 Effect of aminophospholipids on L_o/L_d micron-scale immiscibility. Control GUVs were composed of 60/20/20 eggPC/eggSM/CHOL mixture (a and b). Dark round-shaped L_o domains appeared at 20.3 °C. For clarity, the visualization of L_o domains was always represented at slightly lower temperatures than the temperature of their formation (a). Domain size increased with temperature decrease (4 °C) (b). L_o domain formation when one aminophospholipid is added to the control mixture: 50/10/20/20 eggPC/eggPE/eggSM/CHOL (c and d) and eggPC/brainPS/eggSM/CHOL (e and f). L_o domain formation in the presence of two aminophospholipids: 40/10/10/20/20 eggPC/brainPS/eggPE/eggSM/CHOL (g and h). Scale bar 20 μm

aminophospholipids in the eggPC/brainPS/eggPE/eggSM/CHOL mixture induced L_o/L_d micron-scale immiscibility at the highest temperature compared to single aminophospholipid effect. Larger apparent L_o fraction was observed when two aminophospholipids participated in the mixture (Fig. 3h)

compared to the control (Fig. 3b) but smaller one compared to eggPE-mixture (Fig. 3d) (Table 1).

Based on this study, we were able to visualize that eggPE demonstrated greater propensity to form micron-scale L_o domains than eggPC and brainPS-ones in L_o/L_d exhibiting mixtures. Two mechanisms could be involved to explain larger micron-scale L_o domains in the presence of eggPE: greater degree of fatty acid unsaturation and the size of the polar head. Because eggPE is the most unsaturated molecule species in this glycerophospholipid group it is unlikely its direct participation in the building of the L_o domains compared to brainPS at which there would have lesser constraints to partition in these domains. The presence of more double bonds in PE compared to brainPS results in extensive kinking of acyl chains that causes very poor tight-packing abilities and much lesser miscibility of the molecule in tightly packed ordered domains. Moreover, the small polar head of PE [30] appears to be a steric hindrance for CHOL to mix more easily in such lipid bilayers, as it by itself also has a small polar head (an OH group). According to the “umbrella model” less polar CHOL accommodates under relatively larger polar heads of phospholipids in order to avoid energy costs associated with the unfavorable contact between the non-polar parts of the CHOL molecule and water [31]. Smaller polar head of the PE molecule is less efficient than that of the PC and PS to provide a shielded area for the CHOL polar head. This molecular model is able to explain why CHOL exhibits lesser miscibility in PE species compared to PC ones [31]. In this study, both, higher degree of acyl chain unsaturation and smaller polar head of PE compared to brain PS and eggPC presume an indirect role of eggPE by decreasing the miscibility of CHOL and SM for more disordered phase (L_d) which leads to the formation of larger fraction of L_o domains. It means more SM and CHOL are available to build the L_o domains. The found temperatures of L_o domain formation with and without aminophospholipids in our study are in accordance with the data obtained on the thermal stability of ordered domains in raft-like ternary mixtures [20]. The smaller the polar head and the more unsaturated fatty acid chains of the phospholipid are, the more thermo-stable L_o domains will be.

We speculate that the indirect role of aminophospholipids to enlarge the fraction of L_o domains can be perceived as a trick of nature to compensate poor SM content in the inner leaflet of the cell plasma membrane in order to keep the ability of lipids to form domains in L_o phase. In our previous studies we demonstrated that others sphingolipids, such as ceramide and sphingosine, could make more thermo-stable L_o domains and expand their fractions [32, 10, 11]. Thus, one can assume that even low sphingolipid levels in the inner leaflet of the plasma membrane could serve as a trigger molecule to form liquid-ordered domains. The aminophospholipids decrease the miscibility of sphingolipids and CHOL for liquid-disordered regions and play a role to assemble them in liquid-ordered domains. It's in accordance with the relation found out between the affinity

of cholesterol and glycerophospholipids [6], the lower the cholesterol affinity is, the higher the temperature of L_o/L_d immiscibility will be. Moreover, it has been suggested that there is some mechanism attracting CHOL to the inner leaflet [33, 1]. The authors hypothesized that CHOL is drawn to the inner leaflet to reduce the bending free energy of the membrane caused by the presence of PE. This could be done in two ways: first by simply diluting the amount of PE in the inner leaflet and second by ordering the tails of the PE to reduce its spontaneous curvature. This evidence supports our finding that aminophospholipids have the ability to stimulate the formation of liquid-ordered domains in the inner leaflet.

Conclusion

In this study we visualized by using fluorescence microscopy of giant vesicles that aminophospholipids segregate in leaf-like gel domains in phosphatidylcholine membranes. Cholesterol increased the miscibility of aminophospholipids. Cholesterol and aminophospholipids were not able to form micron-scale liquid-ordered domains as it is an intrinsic property of saturated phosphocholines and CHOL in unsaturated glycerophospholipid matrix. However, in the presence of aminophospholipids, larger liquid-ordered domains were formed in SM-containing raft-like mixtures in the following order: PE+PS>PE>PS>PC. In conclusion, aminophospholipids could regulate the size of the liquid-ordered domains. Further systematic studies on other minor lipids and mostly developing asymmetric model membranes will be needed in order to reveal the mechanism of raft-like formation in the inner leaflet of cell plasma membranes.

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