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EFFECT OF *N*-PROPYL GALLATE ON LIPID PEROXIDATION IN HETEROGENOUS MODEL MEMBRANES

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ABSTRACT

The antioxidant n-propyl gallate (nPG) is widely used in the food industry, pharmaceutics and cosmetics. Therefore, we should be well acquainted with its exact mechanism of action and its effects on the human organism, especially at the molecular level. Because of its slight solubility in water, it could be expected that, in cells, nPG would enter the membrane structures, thus altering their properties. Up to date little is known about its interaction with heterogenous lipid bilayers. That is why we focused our present study on the influence of nPG on the phase behavior of phosphatidylcholine/sphingomyelin/cholesterol (PC/SM/CHOL) ternary mixtures. Fluorescence microscopy of giant unilamellar vesicles (GUVs) was used as an experimental approach. Two phosphatidylcholine species were compared: palmitoyl-oleoyl phosphatidylcholine (POPC) and palmitoyl-docosahexaenoyl (ω-3) phosphatidylcholine (PDPC) differing in the number of double bonds at the sn-2 position (1 for POPC and 6 for PDPC). Fluorescence microscopy observations showed that the presence of docosahexaenoic acid induced the formation of micron-scale liquid-ordered (L_o) domains (model of cellular "rafts") at physiological and higher temperatures compared to the monounsaturated oleic acid. nPG decreased the liquid-ordered (L_o)/liquid-disordered (L_d) miscibility transition temperature, T_m, for both types of vesicles in a concentration-dependent manner. Its effect was more pronounced in GUVs composed of PDPC/SM/CHOL.

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Introduction

n-Propyl gallate (*n*- propyl 3,4,5-trihydroxybenzoate) is a synthetic derivative of the naturally occurring antioxidant gallic acid or its ester form. Its radical scavenging effect is due to gallic acid (3,4,5-trihydroxybenzoic acid), which is a natural component of many plants (2, 8). *n*PG as well as other synthetic derivatives of the galic acid are commonly used as antioxidants in the food industry (28, 29, 35). The compound is poorly soluble in water but is soluble in ethanol, ethyl ether, oil, lard. The biological effects of this antioxidant species include antimicrobial activity, enzyme inhibition, inhibition of biosynthetic processes, inhibition of the formation of nitrosamines, anesthesia, inhibition of neuromuscular response to chemicals, ionizing/ultraviolet (UV) radiation protection, etc. (10). The information about the effect of *n*PG on lipid phase transitions, membrane properties and organization is scarce. There is little available data from fluorescence microscopy studies with model membranes that *n*PG could attenuate artifacts arising from: 1) the excitation of fluorescent probes, which is believed to cause the formation of light-induced liquid-ordered (L_o) domains and 2) the electric fields applied during electroformation, which could oxidize lipid acyl chains (2, 37). L_o phase is characterized with high lateral mobility of lipids and dense packing of fatty acid chains and is considered as a model of cellular domains called lipid "rafts". These domains are enriched in cholesterol, sphingolipids and GPI-anchored proteins (5, 30). It is postulated that "rafts" play an important role in many cellular processes (5).

An interesting component of certain cellular membranes is docosahexaenoic acid (DHA), which is considered to influence membrane organization, and especially that of lipid "rafts", thus modulating cell signaling (32). According to published reports, polyunsaturated fatty acids, PUFAs (containing two or more double bonds), are more prone to oxidation (38). PUFAs which belong to the omega-3 family are essential for human health. The human organism cannot synthesize them, which means they should be taken with the food. Docosahexaenoic acid (DHA) is an ω-3 PUFA that naturally occurs in fish oils (22). In the human organism it is found mainly in brain phospholipids. Its high levels in the brain of mammals were reported for the first time by Thudichum in the 1860s (34). DHA content usually exceeds 50 % of the total amount of acyl chains in certain specialized tissues, for example, synaptosomal membranes (4) and the retinal rod outer segment (36).

Docosahexaenoic acid is known to have a therapeutic value in the treatment of neurodegenerative diseases (e.g. dementia, Alzheimer's disease), cognitive performance in adults, depression, bipolar disorders (6, 21, 25) and many other pathological conditions.

The key to revealing the biological significance of DHA is probably in its unique structure (33). Its chain consists of 22 carbon atoms and has six double bonds. The long unsaturated fatty acid chain of DHA is extremely flexible and has a high degree of molecular disorder. It displays a fast interconversion among several possible conformations. This results from the

lowered energy barrier for rotational isomerization about single C–C bonds, located between the double bonds in PUFA (7, 9). NMR data shows that the remarkable mobility of DHA chains creates membranes that are quite thin and fluid (15, 27), permeable (16) and elastic (31).

The biological significance of this essential fatty acid makes it a preferable subject of our comparative study between monounsaturated and polyunsaturated phosphatidylcholines included in raft-forming ternary mixtures (phosphatidylcholine/sphingomyelin/cholesterol). Special attention was paid to the liquid/liquid phase separation of the target lipids and, particularly, to the effect of lipid oxidation caused by ultraviolet light during fluorescence observation. *n*PG was used as a photo-protector against lipid oxidation. Additionally, in this study we put an emphasis on the influence of this antioxidant molecule on the temperature of liquid/liquid phase separation, domain size and their fraction in biomimetic systems.

Materials and Methods

Materials

L-α-phosphatidylcholine-β-palmitoyl-γ-oleoyl (POPC), L-α-phosphatidylcholine-β-palmitoyl-γ-docosahexaenoyl (PDPC), egg-yolk sphingomyelin (SM) and the fluorescent lipid analogue L-α-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhod-PE) were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Cholesterol (CHOL) was from Sigma–Aldrich (St Quentin-Fallavier, France). The 0.5 mmol/L Hepes buffer, pH 7.4 (conductance σ = 59 μS/cm) and the antioxidant *n*-propyl gallate (propyl 3,4,5-trihydroxybenzoat) (*n*PG) were also purchased from Sigma.

Electroformation and visualization of giant unilamellar vesicles

The GUV samples were prepared using an electroformation method developed by Angelova and Dimitrov (1). The lipid mixtures were prepared in diethyl ether/chloroform/methanol (in a 70/20/10 ratio) at 0.5 mg/mL total lipid. Using a Hamilton micro syringe pipette, about 8 μL of the lipid solution were placed onto the surface of two parallel platinum electrodes, 0.8 mm in diameter each and at 3 mm distance apart. The solvent was evaporated under vacuum and then the electrodes were placed in a temperature-controlled quartz chamber. Vesicles were formed in 0.5 mmol/L Hepes buffer, pH 7.4. For some experiments, *n*PG was added to the buffer phase, dilluted in Hepes buffer to 0.5 mmol/L, 1 mmol/L, 2 mmol/L, 4 mmol/L and 5 mmol/L (final concentration in the quartz chamber). Only fresh *n*PG solutions were used to avoid the formation of *n*PG crystals.

GUVs were observed using a Zeiss Axiovert 135 microscope equipped with 63x long working distance objective lens (LD Achroplan Ph2). Observations were recorded using a Zeiss AxioCam HSm CCD camera connected to an image-recording and processing system (Axiovision, Zeiss). Lipid phase separation in GUVs was followed in fluorescence by Zeiss filter set 15 (Ex/Em = 546/590 nm).

Results and Discussion

As highlighted above, *n*PG is recognized as a powerful antioxidant and added to foods (especially oils and fats), cosmetics and hair products (8). Besides its antioxidant activity, *n*PG displays pharmacological effects such as anti-inflamatory (11) and anti-tumor activities (20). Its precursor, gallic acid, as well as *n*-propyl gallate itself are among the active ingredients found in green tea (13). However, it was established that the hepatotoxic effects of green tea are partly attributed to these molecules. It is summarized that *n*PG causes depletion of cellular ATP and cell death at concentrations above 1 mmol/L (23, 24). Another research group demonstrated that *n*PG and gallic acid exert their radical scavinging effects at concentrations up to 200 μmol (18). In tumor cell lines 786A, TA3 and TA3-MTX-R, *n*PG produces inhibition of mitochondrial function at very low concentrations. Furthermore, it was discovered that *n*PG has neuroprotective effects, using a model of forebrain ischemia in rats (12, 17). These investigations well demonstrate that when *n*PG is used in various products, its concentration should be taken into account. As far as we are acquainted, its concentration in cosmetic products, for example, should not exceed 1 % (10).

We speculate that, in cells, *n*PG probably enters the membrane structures because of its poor solubility in water. It is interesting that another group of free-radical scavengers – flavonoids – are reported to interact with membranes. They cause changes in membrane physical properties and influence the rates of membrane lipid and protein oxidation. The authors state that polyphenols participate in hydrogen bonds at the surface of bilayers and, thus, they reduce the access of oxidants to the membrane (26). The influence on membrane properties could be a commmon feature in the mechanism of action of lipophilic antioxidants. However, no detailed information about the interaction of *n*PG with membranes is available. Such an interaction was proposed for the first time by Zhao et al. (37) based on their fluorescence microscopy observations, which will be discussed in detail below.

Besides its many other applications, *n*PG also has an important role in fluorescence microscopy studies. Recently, it has been reported that the intense illumination during vesicle electroformation can induce L_o/L_d phase separation in lipid mixtures that contain a fluorescent dye. The exact mechanism of light-induced domain formation, however, remains unknown. It has been proposed by Ayuyan and Cohen (2) that lipid peroxides and their breakdown products are the main participants in this event. Zhao et al. (37) suggest the possibility that lipids can polymerize, as it was described for the drying and oxidative degradation of linseed oil (19). Ayuyan and Cohen (2) also reported that the free radical scavenger *n*PG was able to reduce artifacts arising from photooxidation.

In light of the experimental evidence discussed above, we compared GUVs composed of 50/25/25 (mol/mol/mol)

POPC/SM/CHOL and PDPC/SM/CHOL, in order to study the influence of the degree of unsaturation at the *sn*-2 position in phosphatidylcholines on liquid-ordered (L_o)/liquid-disordered (L_d) phase separation. In both types of vesicles we followed the phase changes occurring upon the addition of *n*PG to the buffer phase. GUVs were monitored by video fluorescence microscopy in the temperature range of 60 °C to 4 °C.

POPC-containing mixtures yielded homogenous vesicles from 60 °C to 24 °C. At 23.4 °C (±2.4 °C) small dark domains on a bright background appeared (**Fig. 1a**). The dark domains were round-shaped and quickly grew in size through fusion. Therefore, we assume they existed in the liquid-ordered (L_o) phase state. The bright domains probably existed in liquid-disordered phase state (L_d), while the surrounding darker phase was in liquid-ordered (L_o) state, as it is known that the fluorescent dye, Rhodamine-PE, preferentially partitions into the more disordered L_d phase (3). POPC vesicles showed phase percolation from 13 °C to 4 °C, many bright round domains on a dark background were observed (**Fig. 1b**). There was no alteration in the size of the bright domains even at temperatures as low as 5 °C (**Fig. 1c**).

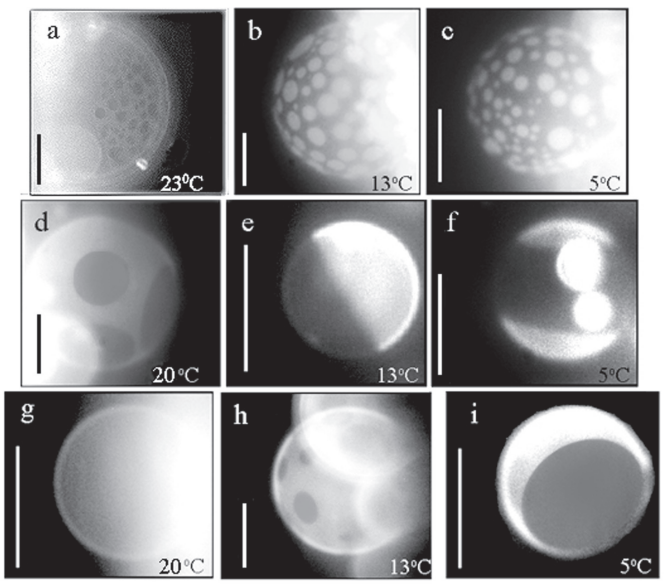

Fig. 1. L_o/L_d phase separation in GUVs composed of 50/25/25 POPC/SM/CHOL ternary mixtures without *n*PG (a–c), and in the presence of 1 mmol/L (d–f) and 5 mmol/L *n*PG (g–i). The fluorescent marker Rhod-PE was used at a concentration of 1 mol %. Scale bars are 20 μm.

In PDPC/SM/CHOL vesicles, phase separation was detected at considerably higher temperatures (49.8 °C ± 2.0 °C). These vesicles displayed dark domains on a bright background near the physiological temperature (**Fig. 2a**). If vesicles were left long enough (e.g. for about 1 h) at constant temperature to reach a steady state, the dark domains increased their size through fusion until one large domain developed, occupying 1/2 or 1/3 of the vesicle surface (**Fig. 2b**). Such large domains also formed upon cooling (**Fig. 2c**).

For both POPC- and PDPC-containing mixtures, *n*PG induced a gradual decrease in the micron-scale miscibility transition temperature, T_m, when applied at concentrations above 1 mmol/L. In **Fig. 1** and **Fig. 2** (**d–f**) it is clearly seen that 1 mmol/L *n*PG had no visible effect on T_m. Contrary to PDPC vesicles, POPC-containing ones displayed a change in phase morphology. At 1 mmol/L *n*PG, dark L_o domains surrounded by a bright L_d phase were observed in POPC/SM/CHOL vesicles compared with the control mixture without *n*PG (**Fig. 1d**). Upon decreasing the temperature, these domains merged and reached about 1/2 of the vesicle surface (**Fig. 1e**). At very low temperatures (~5 °C) the L_o phase was predominant (**Fig. 1f**). Such a change in phase morphology was not detected for PDPC mixtures (**Fig. 2d–f**). At a concentration of 5 mmol/L, *n*PG shifted the domain formation temperature to lower values. POPC vesicles were homogenous at 23 °C (data not shown), unlike the control (**Fig. 1a**). The same was observed in PDPC vesicles: they were homogenous at 49.8 °C (data not shown) in contrast to the control, where dark domains were detected at the same temperature. In the presence of 5 mmol/L *n*PG, POPC-containing vesicles were homogenous from 60 °C to 14 °C (**Fig. 1g**). They displayed dark L_o domains surrounded by an L_d phase at about 13 °C (**Fig. 1h**). Upon cooling, these domains merged and one large domain occupying 1/2 or 1/3 of the vesicle surface formed at 5 °C (**Fig. 1i**), similar to GUVs containing PDPC (**Fig. 2c, f, i**).

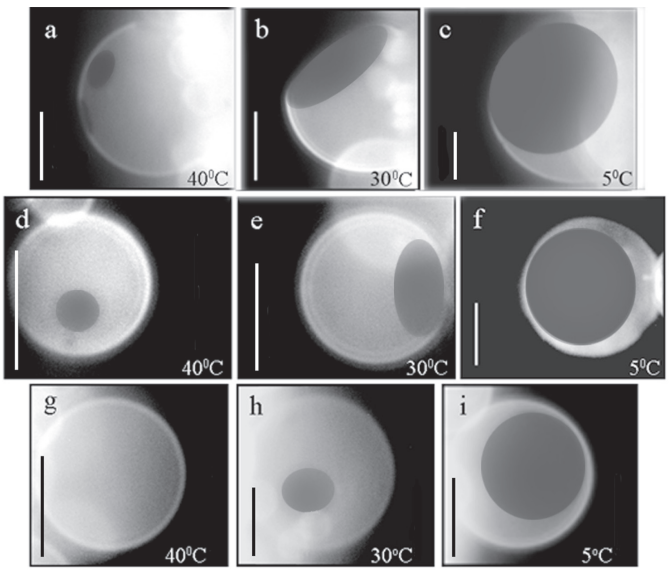

Fig. 2. L_o/L_d phase separation in GUVs composed of 50/25/25 PDPC/SM/CHOL ternary mixtures without *n*PG (a–c), and in the presence of 1 mmol/L (d–f) and 5 mmol/L *n*PG (g–i). The fluorescence marker Rhod-PE was used at a concentration of 1 mol %. Scale bars are 20 μm.

To summarize the results presented above, *n*PG largely affected the T_m in GUVs composed of PC/SM/CHOL mixtures, as well as the vesicle size. The dependence of T_m on the concentration of *n*PG for 50/25/25 POPC/SM/CHOL and PDPC/SM/CHOL vesicles is presented in **Fig. 3**. The miscibility temperature decreased with increasing the *n*PG concentration in the buffer solution. The effect was more significant for PDPC than for POPC mixtures. A great

reduction in vesicle size upon increasing the amount of *n*PG was detected for both POPC- and PDPC-containing vesicles.

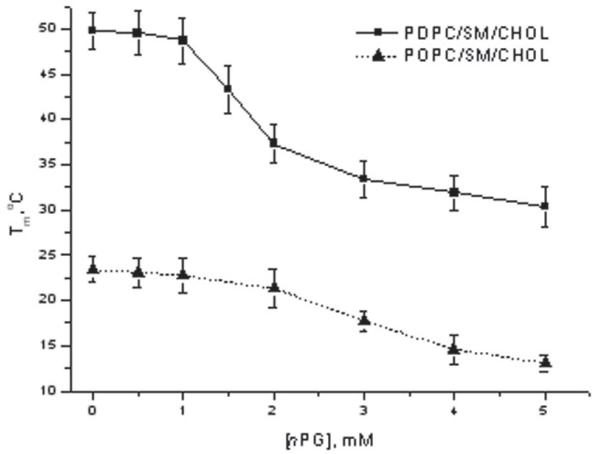

Fig. 3. L_o/L_d micron-scale miscibility transition temperature (T_m) of GUVs prepared from 50/25/25 POPC/SM/CHOL and PDPC/SM/CHOL ternary mixtures as a function of the concentration of *n*PG.

Based on our fluorescence microscopy study, we propose that the decrease in the temperature of domain formation when *n*PG > 1 mmol/L (**Fig. 1** and **Fig. 2d–f**) might be a phase behavior effect due to the presence of an additive component in the lipid membrane bilayer. The last argument supports the idea of Zhao et al. (37), who suggest that *n*PG might enter the membrane bilayer. We suggest that *n*PG could play a photoprotective role at concentrations as low as 0.5 mmol/L to 1 mmol/L because light-induced L_o/L_d phase separation appeared after 10 min of continuous UV exposure. Such time duration is large enough, when the experimenter uses only the images taken with total UV exposure time up to 2 s. Moreover, we always observed the vesicles from the top of the electrodes to the bottom, with maximally closed aperture, to avoid the illumination of other vesicles. Our results are in agreement with the findings of other authors (2, 37) who recently reported that *n*PG added to GUV preparations inhibited the formation of light-induced domains. However, these reports do not discuss what the threshold concentration of *n*PG which impacts the intrinsic phase behavior of the mixture is. By using differential scanning calorimetry, we showed that *n*PG exhibited a strong fluidizing effect on sphingomyelin and both types of mixtures, POPC- and PDPC-containing ones. *n*PG at a concentration of 5 mmol/L was able to completely abolish the phase transition of sphingomyelin (14).

As we already stated, 1 mmol/L of *n*PG could be such a threshold concentration at which the photoprotective role of *n*PG dominates over its phase-modulating effect during UV exposure duration not exceeding 10 min (only if a 50 W UV lamp is used). We demonstrated how the presence of 1 mmol/L *n*PG could change the phase morphology of co-existing phases in a POPC/SM/CHOL mixture as compared to the control without *n*PG: from bright L_d domains on dark L_o background to L_o domains on L_d background (**Fig. 2a–f**). Additionally, the temperature of the L_o/L_d phase separation still remains within the confidence intervals (±2.4 °C).

Conclusions

The results from our study showed that *n*PG can influence the membrane lateral organization, especially the properties of "rafts". It decreases the temperature of liquid-ordered (L_o)/ liquid-disordered (L_d) phase separation, the effect of *n*PG being more pronounced for polyunsaturated PC/SM/CHOL than for monounsaturated PC/SM/CHOL ternary mixtures. Because of the wide range of application of *n*PG and its important biological role, further investigations should be performed to reveal its exact mechanism of membrane antioxidant protection and direct participation in the anti-inflamatory and anti-tumor activities.

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