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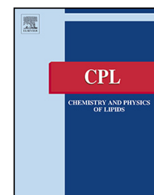

Short communication

Effect of flavonoids on the phase separation in giant unilamellar vesicles formed from binary lipid mixtures

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ABSTRACT

Confocal fluorescence microscopy have been employed to investigate phase separation in giant unilamellar vesicles prepared from binary mixtures of unsaturated dioleoylphosphocholine with saturated phosphocholines or brain sphingomyelin in the absence and presence of the flavonoids, biochanin A, phloretin, and myricetin. It has been demonstrated that biochanin A and phloretin make uncolored domains more circular or eliminate visible phase separation in liposomes while myricetin remains the irregular shape of fluorescence probe-excluding domains. Influence of the flavonoids on the endotherms of liposome suspension composed of dioleoylphosphocholine and dimyristoylphosphocholine was investigated by the differential scanning calorimetry. Calorimetry data do not contradict to confocal imaging results.

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1. Introduction

Phase separation in cellular membranes is thought to play a critical role in a variety of biological functions, such as signal transduction, cytoskeletal organization, protein trafficking/recycling, and cell–cell communication (Laux et al., 2000; Moffett et al., 2000; Simons and Toomre, 2000; Simons and Ikonen, 1997; Kwik et al., 2003; Young et al., 2005; Pierchala et al., 2006; Landry and Xavier, 2006). Under normal physiological conditions, lipid domains in the cellular membranes are sub-microscopic and dynamic. For this reason, it is difficult to demonstrate the existence of different lipid phases in the membranes of living cells. The study of the physical and chemical properties of artificial lipid systems, where the lipid composition and environmental conditions (such as temperature, ionic strength, and pH) can be systematically controlled, provides more information about the behavior of the lipid matrix. Giant unilamellar vesicles (GUV) are a perfect tool for visualizing phase separation in model systems (Wesołowska et al., 2009a). The existence of at least three lipid phases in model membranes is suggested: a liquid ordered (*lo* or 'rafts') phase, a liquid disordered (*ld*) phase, and a solid ordered (*so*) or gel phase. Pure phospholipid bilayers exist in either the *so* or the *ld* phase, depending on whether the temperature is below or above the melting temperature (T_m) for that lipid. Model membranes containing high and low T_m phospholipids may exhibit coexistence of *so* and *ld* phases. It is believed that sterol, in particular cholesterol, is an absolute requirement for the formation of the intermediate *lo* phase (Sankaram and Thompson, 1990). Investigation of phase separation may be performed by using fluorescent lipid probes that have different partition coefficients between lipid phases. Many labeled lipids prefer the *ld* phase, causing ordered domains to remain uncolored (Juhasz et al., 2010). The shape of the lipid domain correlates with its physical properties. In particular, circular domains are usually attributed to the *lo* phase, while non-circular and dendritic domains are usually attributed to the *so* phase (Bagatolli and Graton, 2000; Samsonov et al., 2001; Veatch and Keller, 2003; Bagatolli and Kumar, 2009; Muddana et al., 2012). Concentration-dependent ability of labeled lipid alters phase coexistence should be taking into account. For example 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) in 0.1 mol% raises phase transition temperature by 2.5 °C in certain *lo* containing lipid mixtures (Juhasz et al., 2010).

Flavonoids are a class of polyphenols that are found ubiquitously in plants. They possess significant biological activity related to their antioxidant, anti-allergic, anti-inflammatory, antimicrobial, and anticancer properties (Yamamoto and Gaynor, 2001; Cushnie and Lamb, 2011; Hendrich, 2006; de Sousa et al., 2007). In vitro studies indicate that some flavonoids alter lipid packing (Ollila et al., 2002; Tarahovsky et al., 2008) and decrease the membrane dipole potential (Cseh and Benz, 1998; Efimova and Ostroumova, 2012).

The present study is devoted to investigation of effects of plant flavonoids on phase segregation in the membrane. Three flavonoids, biochanin A, phloretin and myricetin, were chosen to

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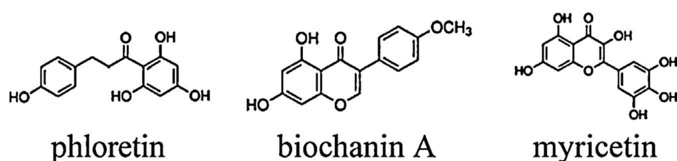

Fig. 1. The chemical structures of flavonoids, phloretin, biochanin A, and myricetin.

test their effects in two-component lipid bilayers made from high and low T_m lipids.

2. Materials and methods

2.1. Materials

All chemicals were of reagent grade. Synthetic 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), brain sphingomyelin (SM), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DPPE) were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Biochanin A (5,7-Dihydroxy-4′-methoxyisoflavone), phloretin (3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxy-phenyl)-1-propanone), and myricetin (3,3′,4′,5,5′,7-Hexahydroxyflavone) were purchased from Sigma Chemical (St. Louis, MO). The chemical structures of flavonoids are presented in Fig. 1.

2.2. Guv preparation

Giant unilamellar vesicles (GUV) were formed by the electroformation method on a pair of indium tin oxide (ITO) slides with using a commercial Nanion vesicle prep pro (Munich, Germany). Lipid stock solutions at a concentration of 11 mM were prepared in chloroform. Labeling was carried out by addition of the appropriate amount of the fluorescent lipid probe. Rh-DPPE concentration in each sample was 1 ± 0.1 mol%. 18 μl of lipid stock solution was placed on the ITO slide in the center of O-ring. Solvent evaporation was allowed to proceed for a few minutes before 250 μl of 0.5 M aqueous sorbitol solution was added to the dry lipid film and covered with another ITO slide. Alternating voltage with an amplitude of 3 V and a frequency of 10 Hz was applied across the ITO slides for 1 h. GUV formation was carried out at room temperature (25 ± 1 °C). After this, the upper ITO slide was removed and 250 μl of GUV suspension containing 0.8 mM lipid was vigorously taken away. The resulting GUV suspension was divided into 50 μl aliquots. 0.5 μl of 40 mM of ethanol stock solution of flavonoid was added to aliquot. The GUV suspension with flavonoid was allowed to equilibrate for 15 min at room temperature. The final flavonoid concentration in the sample was 400 μM and ethanol concentration was 1% (v/v). It is known that alcohols reduce melting temperature of fully hydrated DPPC (Ohki et al., 1990). Since in our experiments ethanol was used to obtain stock solution of flavonoids we additionally checked if there was any significant influence of this compound on phase separation scenario. Addition of ethanol at the 1% (v/v) caused no change in phase behavior. For estimation of influence of large concentration of labeled lipid on the phase behavior GUV contained 0.1 and 1 mol% of Rh-DPPE were compared. There were not sighted any difference between them except brightness of GUV. Since fluorescence intensity of GUV at 0.1 mol% molar fraction of labeled lipid did not allow to distinguish colored and uncolored regions in the membrane due to quenching by myricetin finally 1 mol% of Rh-DPPE was chosen for the experiments.

Liposomes for differential scanning calorimetry (DSC) experiments were formed from 80 mol% DMPC and 20 mol% DOPC without Rh-DPPE by the method described above with some variations. Lipid stock solution had a concentration of 14 mM in chloroform. 65 μl of lipid stock solution was placed on ITO slide. After the solvent has been evaporated the formed lipid film was rehydrated by 250 μl of distilled water and covered by another ITO slide. Alternating voltage with an amplitude of 3 V and a frequency of 100 Hz was applied across the ITO slides for 1 h in temperature 37 °C. Finally, 250 μl of GUV suspension was diluted to 1 ml by distilled water up to final lipid concentration 0.93 mM. Then, 10 μl of 40 or 200 mM of ethanol stock solution of flavonoid was added to suspension up to concentration of 400 or 2000 μM, respectively.

2.3. Imaging and lipid phase discrimination

The sample was a standard microscopy preparation. 10 μl of the resulting GUV suspension was placed on a standard microscope slide and covered by a coverslip. The suspension occupied the 3-dimensions space with 2 cm × 2 cm × 25 μm (length × width × height, respectively). GUV were located without deformation. We did not observe drying up of the suspension during one hour–a maximum time of sample observation. GUV were imaged through an oil immersion objective 100×/1.4 HCX PL in Leica TCS SP5 confocal laser system Apo (Leica Microsystems, Mannheim, Germany). Temperature during observation was controlled by the air heating/cooling in the thermally insulated camera. Rh-DPPE was excited at wavelengths of 543 nm (helium–neon laser). The pixel size was 0.5 μm. Sample imaging was performed at room temperature (25 ± 1 °C).

Rh-DPPE clearly favors liquid disordered phase and it is excluded from gel and liquid ordered phases (Juhasz et al., 2010). This fact does not allow to directly distinguish *lo* and *so* domains because both of them stand uncolored (Baumgart et al., 2007; Juhasz et al., 2010). Discrimination between two ordered phases can be made by taking into account difference of their morphology. Qualitative consideration suggests that the shape of domain is determined by the dynamic balance between two factors: the line tension of domain boundary and bending elasticity. Domination of the line tension leads to a formation of right circular domains due to tendency to minimization of length of domain boundary. So, circular domains may be attributed to the liquid–liquid coexistence within a membrane (García-Sáez et al., 2007; Samsonov et al., 2001; Chiantia et al., 2006; Deitrich et al., 2001; Weis and McConell, 1984; Lee et al., 2011). In contrast with liquid domains solid ordered domains have irregular dendritic form (Bagatolli and Graton, 2000; Wesołowska et al., 2009a; Bagatolli and Kumar, 2009). That is why a conclusion about gel–liquid or liquid–liquid coexistence can be made from the fluorescence images based on morphology of domains (Muddana et al., 2012; Veatch and Keller, 2003). Following this approach GUV within single field of view were divided into three different populations: (1) homogenous liposomes (without visible phase separation) – *ld*; (2) liposomes with circular uncolored domains – *lo*; (3) liposomes with non-circular dendritic uncolored domains – *so*. Several neighboring fields of view were analyzed. Percentage of vesicles (p_i) with the respective kind of the phase separation at each tested system was calculated as the ratio of phase-separated or homogenous GUV to the total number of GUV:

$$p_i = \frac{N_i}{N_t} \cdot 100\%$$

where *i*-type of domain in GUV, it may mean *ld*, *ld* + *lo* or *ld* + *so*, N_i – number of vesicles with *i*-th type of domain (from 0 to 50), N_t – total number of counted vesicles in sample (typically 50). The values of p_i which present data of at least 9 independent experiments were performed on diagrams. Non-bilayers objects were not taking into account.

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