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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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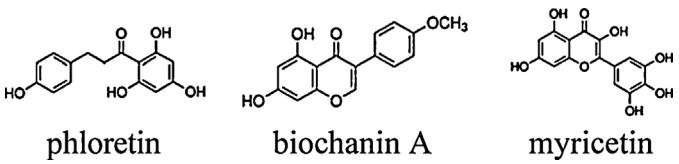

Fig. 1. The chemical structures of flavonoids, phloretin, biochanin A, and myricetin.

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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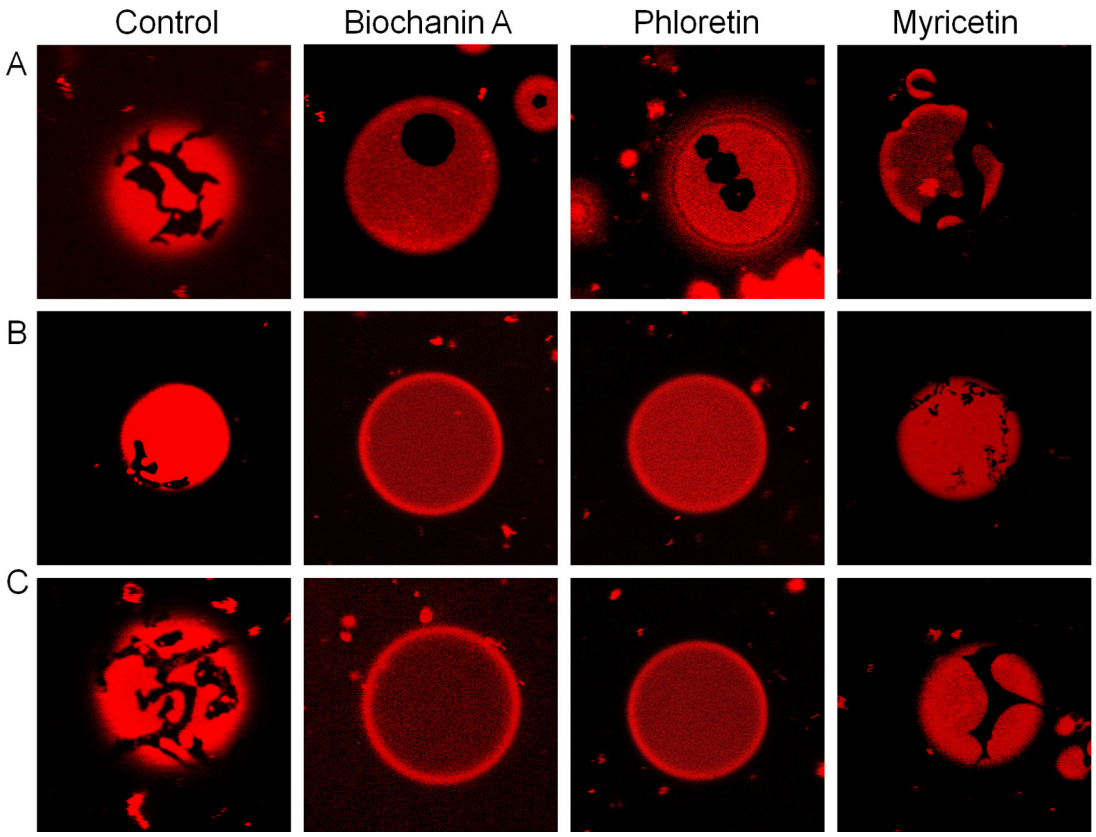

Fig. 2. Fluorescence micrographs of GUV membranes made from DOPC and 20 mol% SM (A), 50 mol% DMPC (B), and 50 mol% DPPC (C) in the absence and in the presence of 400 μM of biochanin A, phloretin, and myricetin in bilayer bathing solutions. The size of each image is 26 μm × 26 μm.

2.4. Differential scanning calorimetry measurements

Differential scanning calorimetry (DSC) experiments were performed using a DASM-4 differential scanning microcalorimeter ("Biopribor", Pushchino, Russia). Appropriate amount of GUV suspension for DSC experiments was heated at a constant rate of 1 K/min and a constant pressure of 2.4 atm. The reversibility of the thermal transitions was assessed by reheating the sample immediately after the cooling step from the previous scan. The thermal transition curves were baseline corrected by subtracting scan of the pure water or water-flavonoid solution in suitable concentration in both cells. The excess heat capacity of the sample (C_p) was calculated as described by Privalov and Potekhin (1986). The temperature dependence of the excess heat capacity was analyzed using Origin software (Micro-Cal Inc., Northampton, MA). The thermal behavior of the liposomes suspension was described by the temperature of the maximum of phase transition (T_m) and width at half height (half-width) of peak of the endotherm ($T_{1/2}$).

3. Results

Fig. 2 shows the typical phase separation in GUV made from respective lipid compositions. One can see that GUV composed of DOPC:SM contained *so* domains (Fig. 2A Control). In DOPC:DMPC and DOPC:DPPC lipid compositions similar results were obtained (Fig. 2B and C Control). Thus all tested lipid mixtures exhibited gel/fluid phase coexistence in the absence of flavonoids. Addition of flavonoids into GUV suspension caused altering in phase separation scenario. Biochanin A and phloretin had a tendency to make the domains more circular in DOPC:SM GUV while myricetin did not (Fig. 2A). In GUV from DOPC:DMPC and DOPC:DPPC biochanin A and phloretin predominantly dissolved *so* domains without making *lo*-like ones while myricetin did not provide changing in *so* domains (Fig. 2B and C).

It should be mentioned that mixtures of saturated phosphocholines with DOPC produced a relatively large fraction of homogenous liposomes even at molar concentration 50 mol% vs. 20 mol% in a case of SM. Only homogenous liposomes in the absence of flavonoid were observed in one fifth of the experiments with DOPC:DPPC and DOPC:DMPC. In these cases the addition of biochanin A and phloretin to the GUV suspension did not affect Rh-DPPE distribution in the membranes, whereas myricetin promoted formation of the *so* domains (Fig. 3).

Micrographs show typically encountered GUV. Statistical description of the GUV distribution is given on diagrams (Fig. 4). In the absence of flavonoids in DOPC:SM lipid composition the major population (more than 90%) of the phase separated vesicles which contained *so* domains was observed. Also there were no *lo* ones and less than 10% of *ld* GUV (Fig. 4A). For GUV from DOPC:DMPC and DOPC:DPPC mixtures similar results were obtained. At the same time portion of homogenous liposomes moderately increased up to 20–30% (Fig. 4B and C). In the presence of biochanin A and phloretin number of observed GUV with *so* domains decreased by ~2 times for DOPC:SM and by ~4–7 times for DOPC:DMPC and DPPC:DPPC, also in 40% DOPC:SM GUV and in 10% DOPC:DPPC circular uncolored domains which might be attributed to *lo*-like state were observed (Fig. 4A and B Biochanin A, Phloretin). Myricetin slightly decreased fraction of homogenous GUV in all tested GUV samples (Fig. 4). One can conclude that influence of flavonoids on the phase behavior of bilayer may be divided on fluidization and condensation ways. Biochanin A and phloretin fluidize bilayer while myricetin rather condensate it.

It should be mentioned that addition of flavonoids in concentration of 400 μM to GUV suspension produced strongly non-bilayer

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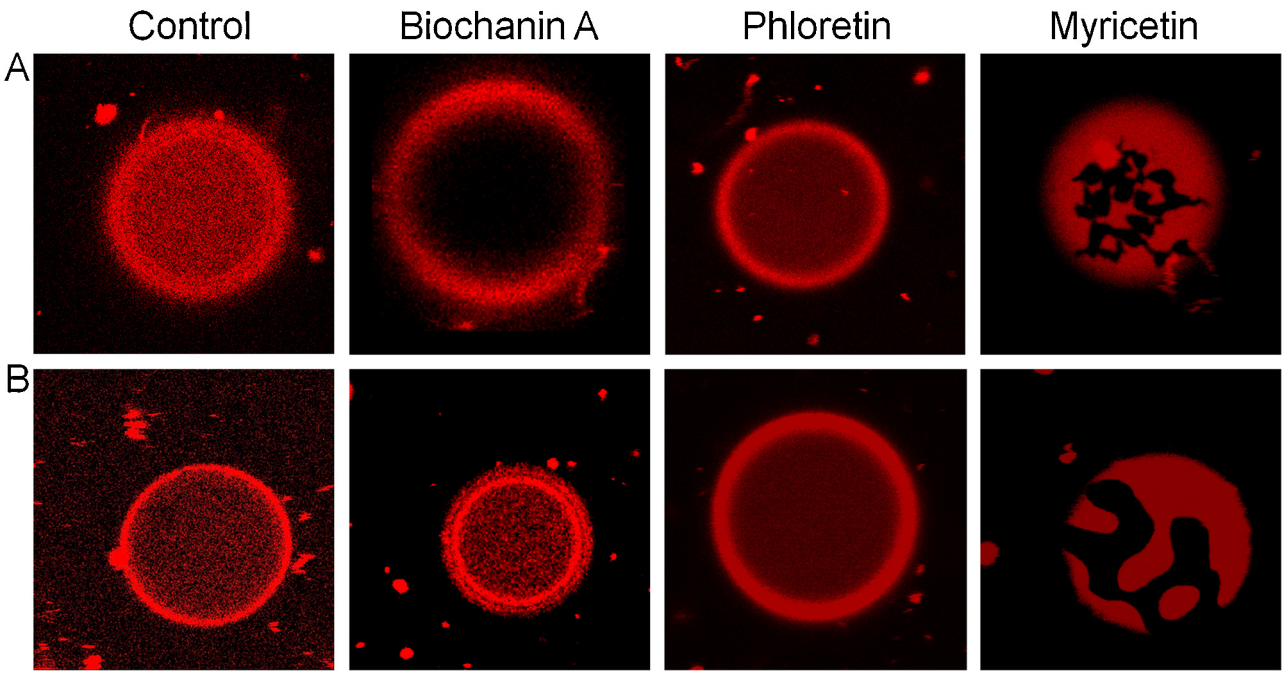

Fig. 3. Fluorescence micrographs of GUV membranes made from DOPC and 50 mol% DMPC (A), and 50 mol% DPPC (B) in the absence and in the presence of 400 μM of biochanin A, phloretin, and myricetin in bilayer bathing solutions. The size of each image is 26 μm × 26 μm. Figure presents the experiments characterized by homogeneous colored GUV membranes in the absence of flavonoids.

cutting needle-shaped structures. It emitted in red part of the spectrum like Rh-DPPE. One can speculate that they may be attributed to lipid-flavonoids complexes. The most presentable ones (DOPC:DPPC suspension in the presence of 400 μM phloretin) are shown in Fig. 5.

Fig. 6 shows endotherms of GUV suspensions. Control GUV suspension containing 20 mol% DOPC and 80 mol% DMPC had transition temperature 15 °C. Addition of 400 μM phloretin to DOPC:DMPC GUV suspension led to significant reduction of the transition temperature $\Delta T_m = -8$ °C compared to control DOPC:DMPC GUV. Transition peak of GUV suspension in the presence of 400 μM myricetin overlapped the peak of DOPC:DMPC liposomes in the absence of flavonoids. Endotherm of GUV suspension in the presence of 2000 μM phloretin had an additional transition peak in a range of 40–50 °C (Fig. 6B) that may be attributed to melting of the non-bilayer structures.

4. Discussion

To understand the role of flavonoids in the formation and dynamics of lipid domains, we analyzed their effects on phase separation in the membranes of GUV made from binary mixtures of low and high T_m lipids. We mixed DOPC ($T_m = -18.3 \pm 3.6$ °C) with DMPC ($T_m = 23.6 \pm 1.5$) °C, DPPC ($T_m = 41.3 \pm 1.8$ °C) (Koynova and Caffrey, 1998), or brain SM ($T_m = 37$–45 °C) (Shaw et al., 2012). Solid ordered domains in the DOPC:DMPC mixture were observed in temperature higher than phase transition temperature of DMPC. The reason for these facts could be the presence of labeled lipid in the membrane. It is known that Rh-DPPE raises mixing/demixing temperature in certain lipid compositions (Juhasz et al., 2010), for example for DOPC:DPPC:cholesterol Rh-DPPE at 0.1 mol% increases demixing temperature by 2.5 °C. At the same time peak of endotherm of liposomes suspension composed of 20 mol% DOPC and 80 mol% DMPC has transition temperature lower than pure DMPC. The peak on thermogram of binary mixture of high and low T_m lipids moves to low temperatures (Fidorra et al., 2009).

Consideration of chemical structure of tested flavonoids admits to differentiation of them. Firstly, biochanin A, phloretin, and myricetin have different number of OH-groups: 2, 4, and 6, respectively (Fig. 1). The feature determines relative hydrophobicity of flavonoids (van Dijk et al., 2000; Scheidt et al., 2004; Oteiza et al., 2005). Ollila et al. (2002) showed that the most hydroxylated flavonoids (e.g., myricetin) have no uncoupling effect on the vesicle membrane while relatively more hydrophobic ones significantly increase calcium release from liposomes. At the same time retention delay on a dynamically coated DPPC column is fivefold larger for myricetin than for apigenin which contains 3 OH-groups (Ollila et al., 2002). The nuclear magnetic resonance study indicates that flavonoids with low number of OH-groups have the diffusion coefficients similar to lipid and they are rather incorporated into lipid membrane while myricetin with 6 OH-groups shows a significantly faster diffusion that is more comparable to membrane associated water molecules (Scheidt et al., 2004). Moreover octanol–water partition coefficients of flavonoids also have reverse correlation with number of OH-groups (Rothwell et al., 2005). Secondary, biochanin A (except a methyl-group) and myricetin have all carbon atoms in sp^2 hybridization and, therefore, they have rigid planar structures while phloretin, which has two carbon atoms in sp^3 hybridization have numerous conformations (Cseh et al., 2000).

Fluidization effect of the biochanin A on the membrane may be explained by its relatively large hydrophobicity. The presence of only two OH-groups allows it to deeper penetrate into hydrophobic core. Also close placement of an OH-group to the adjacent carbonyl facilitates them to form intramolecular hydrogen bond, thereby decreasing in hydrophilicity. In genistein, the closest analog of biochanin A, the same hydrogen bond is formed at the 95% of the time. According to Raghunathan et al. (2012) genistein generally softens membrane. Its long axe is parallel to bilayer plane near the boundary between hydrophobic core and polar headgroups of lipids (Raghunathan et al., 2012). This location probably leads to the increasing of available space for motion of acyl chain and consequently disrupts gel ordered domains. Acacetin, the isomer of biochanin A, reduces T_m of pure DMPC or DPPC liposomes

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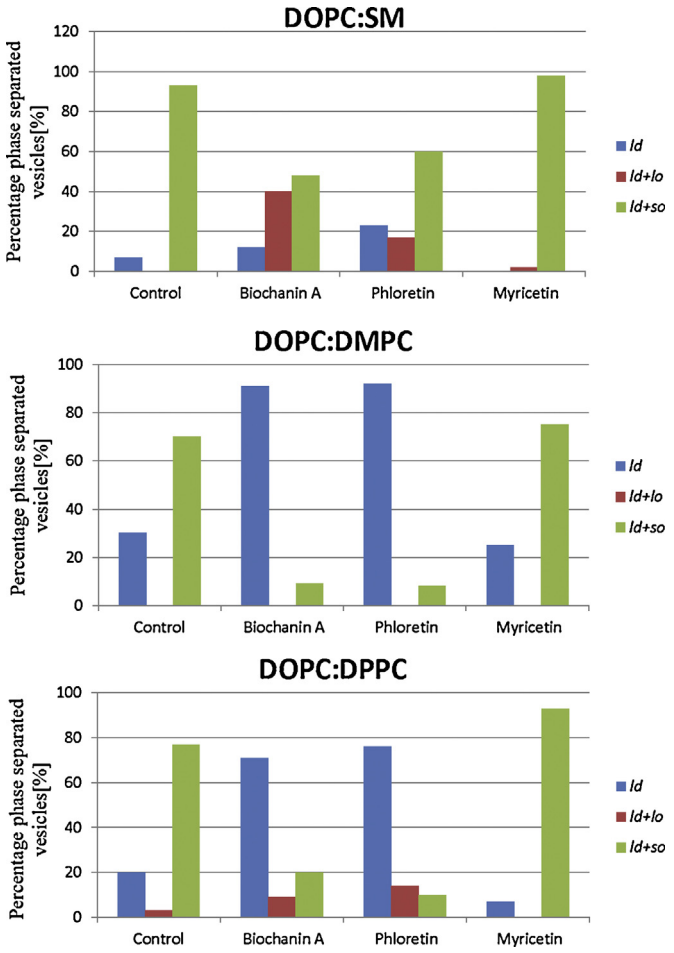

Fig. 4. Diagrams with the percentage distribution of the GUV membranes made from DOPC and 20 mol% SM, 50 mol% DMPC, and 50 mol% DPPC (from top to bottom) in the absence and in the presence of 400 μM of biochanin A, phloretin, and myricetin in suspension (from left to right).

(Wesołowska et al., 2009a). These data support the conclusion for fluidization of membrane in the presence of biochanin A.

Decreasing in T_m of liposomes in the presence of phloretin was showed in pure DMPC vesicles (Cseh et al., 2000; Tarahovsky et al., 2008) and pure DPPC vesicles (Valenta et al., 2004; Auner et al., 2005). We demonstrated that the addition of phloretin at concentration of 400 μM led to significantly reducing of the transition temperature $\Delta T_m = -8\,^{\circ}C$ (Fig. 6A) of GUV composed of 20 mol% DOPC and 80 mol% DMPC. These data indicate that the addition of phloretin to GUV suspension increases entropy of system at given temperature and therefore decreases its ordering. The effect

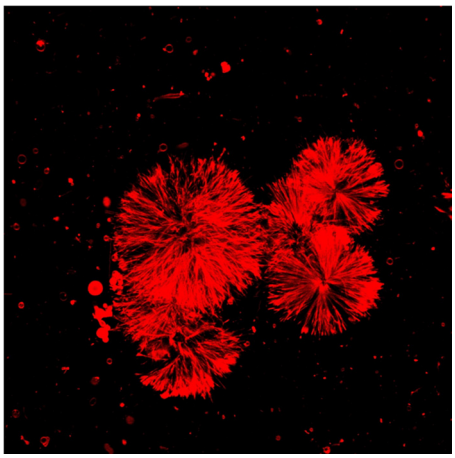

Fig. 5. Non-bilayer structures in the GUV suspension composed of DOPC and DPPC in the presence of 400 μM phloretin. Image size is 160 μm × 160 μm.

of phloretin on solid ordered domains may be accompanied by the formation of fluid compartments where the lipid melting temperature is decreased. So, high T_m lipid rich regions (*so* domains) become liquid ones. Reinl and Bayerl (1993) showed that the integration of hydrophobic molecules in the hydrophobic core leads to a large broadening of the peak of endotherm. Direct intercalating of phloretin in the hydrophobic core is denied by the absence of enlarging in $T_{1/2}$ (Reinl and Bayerl, 1993; Fig. 6A). Phloretin molecules are probably localized within head-group region (Cseh et al., 2000). Data of Tarahovsky (2009) support this assumption. He has calculated possible 3D-model of molecular structure of a complex between phloretin and a phospholipid. Interaction of phloretin with a lipid head-group may lead to increase of the area per lipid molecule and consequently to enhancing of chain mobility.

Myricetin has 6 OH-groups and it is the most hydrophilic one among tested flavonoids. Wesołowska et al. (2009b) showed that myricetin and its close analog quercetin intensively quench fluorescence of head labeled fluorescent lipids. We have observed the same effect in our experiments. The quenching of fluorescence occurs when π-bonds of quencher is close to chromophore of label that proves idea about head-group localization of this compound. 6 OH-groups allow it to form relatively larger numerous of hydrogen bonds with the head-groups oxygens. In contrast with phloretin, which supposed to interact with one lipid molecule, myricetin due to its planar rigid structure may form hydrogen bonds with few neighbored head-groups of lipid. This may generally decrease mobility of lipid molecule and consequently promote the formation of domains. In supporting this idea myricetin has the largest retention delay in DPPC coated column of certain flavonoids and the smallest retention delay in reversed-phased columns

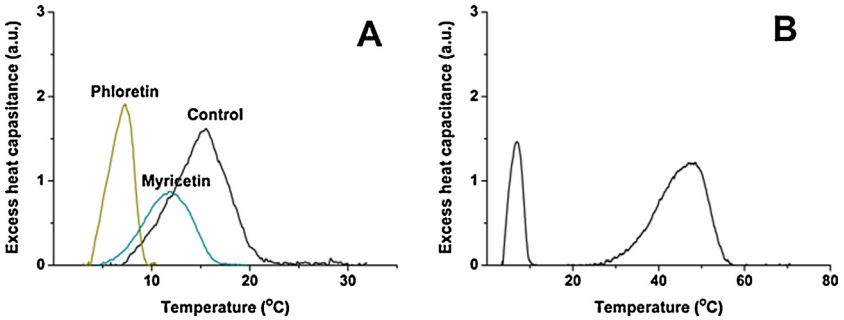

Fig. 6. Endotherms of the liposomes made from 20 mol% DOPC and 80 mol% DMPC: (A) in the absence of flavonoids (control), in the presence of 400 μM phloretin, in the presence of 400 μM myricetin; (B) in the presence of 2000 μM phloretin.

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(Ollila et al., 2002). From nuclear magnetic resonance studies Scheidt et al. (2004) conclude that myricetin apparently is weakly bound by the membrane and able to perform a faster diffusion of higher dimensionality similar to the weakly bound water molecules on the lipid surface. Thermograms of liposomes in the presence of myricetin overlapped ones in the absence of it (Tarahovsky et al., 2008; Fig. 6A) that at least does not contradict this assumption.

5. Conclusions

There is an evidence of the influence of plant flavonoids on the domain morphology in binary lipid mixtures of high and low T_m lipids. Biochanin A and phloretin make Rh-DPPE-excluding domains more circular or eliminate visible phase separation while myricetin does not significantly alter lateral heterogeneity. Effects of flavonoid on the lipid packing correlate with hydrophobicity and flexibility of flavonoid molecules. More hydrophobic biochanin A deeper intercalates in bilayer and fluidizes membrane whereas less hydrophobic myricetin weakly bound with the membrane and does not significantly alter phase separation. Phloretin decreases transition temperature and facilitates destruction of ordered domains probably due to high flexibility, which allows it to strongly interact with a phosphate head-group of lipid. These results suggest that nonlipid amphiphiles contribute to phase separation. Further studies are required to determine whether these flavonoids can tune lipid domain formation in cell membranes. Such behavior would have a significant impact in understanding lipid phase control of cellular biochemical and mechanobiological signaling.

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