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Calcium-dependent aggregation and fusion of phosphatidylcholine liposomes induced by complexes of flavonoids with divalent iron

Yury S. Tarahovsky [a,b,*], Elena A. Yagolnik [c], Eugeny N. Muzafarov [c,d], Bolatbek S. Abdrasilov [e], Yuri A. Kim [f]

[a] Institute of Theoretical and Experimental Biophysics, RAS, Pushchino, Moscow Region, 142290, Russia
[b] Research-Educational Center "Bionanophysics," Moscow Institute of Physics and Technology, 141700 Dolgoprudniy, Russia
[c] Tula State University, Tula, 300000, Russia
[d] Institute of Basic Biological Problems, RAS, Pushchino, Moscow Region, 142290, Russia
[e] Ministry of Education and Science, Astana, 010000, Republic of Kazakhstan
[f] Institute of Cell Biophysics, RAS, Pushchino, Moscow Region, 142290, Russia

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ABSTRACT

It was found that complexes of the flavonoids quercetin, taxifolin, catechin and morin with divalent iron initiated an increase in light scattering in a suspension of unilamellar 100 nm liposomes. The concentration of divalent iron in the suspension was 10 μM. Liposomes were prepared from 1-palmitoyl-2-oleoylglycero-3-phoshpatidylcholine. The fluorescent resonance energy transfer (FRET) analysis of liposomes labeled with NBD-PE and lissamine rhodamine B dyes detected a slow lipid exchange in liposomes treated with flavonoid-iron complexes and calcium, while photon correlation spectroscopy and freeze-fracture electron microscopy revealed the aggregation and fusion of liposomes to yield gigantic vesicles. Such processes were not found in liposomes treated with phloretin because this flavonoid is unable to interact with iron. Rutin was also unable to initiate any marked changes because this water-soluble flavonoid cannot interact with the lipid bilayer. The experimental data and computer calculations of lipophilicity (cLogP) as well as the charge distribution on flavonoid-iron complexes indicate that the adhesion of liposomes is provided by an iron link between flavonoid molecules integrated in adjacent bilayers. It is supposed that calcium cations facilitate the aggregation and fusion of liposomes because they interact with the phosphate moieties of lipids.

1. Introduction

Flavonoids are polyphenolic compounds abundantly constituent in dietary fruits and vegetables. Their daily consumption can reach tens and hundreds milligrams [1]. Depending of diet the concentration of these compounds in blood considerably changes and can reach a few micromoles [2]. The presence of flavonoids in diet is very important for human health because they reveal antiinflammatory [3], antibacterial [4], antiviral [5] and antifungal [6] activity. Flavonoids are also known to prevent cardiovascular [7], cancer [8], and neurodegenerative [9] diseases.

The mechanisms of flavonoid functioning are still not sufficiently studied. It is known that they are able to influence the activity of some enzymes [10,11], or cell signaling processes [12,13] though the main attention is attracted to their antioxidant properties. Flavonoids can protect cells from oxidative stress owing to their radical scavenging activity and also to the ability to helate transient metals including cations of iron and copper [14,15]. Moreover, it was found that flavonoid-metal complexes reveal an elevated radical scavenging activity similar to superoxide dismutase [16].

Lipids of biological membranes are the main targets of oxidative stress. The biological functioning and antioxidative activity of flavonoids correlate well with their lipophilicity and ability to interact with biological membranes [17–19]. The present study is concerned to the interactions of flavonoid-iron complexes with PC liposomes because this subject is scarcely studied. To exclude the influence of lipid oxidative processes on our measurements, we used divalent iron which is less oxidative compared to trivalent iron or copper [15].

2. Materials and methods

2.1. Preparation of liposomes

2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (Sigma-Aldrich) was dissolved in chloroform, dried under argon stream, and vacuumed overnight to remove the traces of organic solvent. The dried lipid film was hydrated in 10 mM Tris–HCl (pH 7.0) to a lipid concentration of

Abbreviations: PC, phosphatidylcholine; POPC, 2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine; DMSO, dimethyl sulfoxide; NBD-PE, [1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)]; Rhodamine-PE, [1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)]; PCS, Photon correlation spectroscopy

* Corresponding author at: Institute of Theoretical and Experimental Biophysics, RAS, Pushchino, Moscow Region, 142290, Russia. Tel.: +7 4867 739266; fax: +7 4967 330553.
E-mail address: tarahov@rambler.ru (Y.S. Tarahovsky).

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15 mg/ml which approximately corresponded to 20 mM. A Mini Extruder (Avanti Polar Lipids, USA) was used for preparation of 100 nm liposomes in accordance with a standard procedure.

2.2. Light scattering measurements

Light scattering was measured at 90° on an MPF-44B spectrofluorometer (Perkin Elmer, USA) at $\lambda = 650$ nm. 20 μL of 20 mM liposomes were added at continuous stirring to 10 mM Tris–HCl buffer (pH 7.0) in a 2 ml cuvette. To prepare stock solutions, rutin was dissolved in water, while taxifolin, catechin, morin, and phloretin were dissolved in 70% ethanol, and quercetin in 50% DMSO. The concentration of all flavonoids was 1 mM. For measurements, 20 μL of flavonoids, 20 μL of 1 mM $FeSO_4$ and 20 μL of 10 mM $CaCl_2$ were added to the cuvette, so that the final concentration of lipids in the cuvette was 2×10^{-4} M. The concentrations of flavonoids and iron were 1×10^{-5} M, of calcium 1×10^{-4} M, of DMSO (present only in samples with quercetin) 5×10^{-6} M. All chemicals were from Sigma-Aldrich (USA). In the control measurements, we did not reveal any influence of DMSO or ethanol amounts used on the samples in all experiments presented in this study.

2.3. Liposome size measurements

The size of liposomes was determined with Photon Correlation Spectroscopy (PCS) on an N4 PLUS Submicron Particle Size Analyzer (Beckman Coulter, USA) equipped with a size distribution processor (SDP) that we used for detailed analysis of particle size distribution. We used the same cuvette and the same concentrations of chemicals as in the light scattering experiments. After addition of chemicals to cuvette, we evaluated the light scattering on a spectrofluorometer as described above, to reach the equilibrium, and after that we transferred the cuvette to the Particle Size Analyzer. The whole time of measurements was 30–40 min.

2.4. Fluorescent assay of lipid exchange

For the fluorescent assay, we prepared POPC liposomes containing 0.5% of NBD-PE and 0.5% of rhodamine-PE (Avanti Polar Lipids, USA). Liposomes were prepared according to the procedure described above, except that the concentration of lipid in the suspension was 5 times lower than that of our standard nonfluorescent liposomes. The equal volumes of fluorescent and nonfluorescent liposomes were added to the cuvette so that the final concentration of lipids was equal to that used in light scattering experiments (2×10^{-4} M). The ratio of labeled to unlabeled liposomes was 1:5. Accordingly, the concentrations of flavonoids, iron, and calcium were also equal to those described in our light scattering experiments. Measurements were performed on an MPF-44B spectrofluorometer (Perkin Elmer, USA) at continuous stirring. The excitation wavelength was $\lambda = 500$ nm. The fluorescence of NBD-PE was registered at $\lambda = 537$ nm, while rhodamine-PE fluorescence at $\lambda = 590$ nm. To determine the extent of fusion as a percentage of the total fluorescence change, we introduced 20 to 40 μL of 1 mM triton X100 to the cuvette at the end of measurements.

2.5. Freeze-fracture electron microscopy

For electron microscopy, we mixed the same volumes of all chemicals as it was described for light scattering experiments, except that they were not diluted in 2 ml of buffer. After 10–20 min incubation at room temperature, the suspension of liposomes was placed between 100 μ thick copper sheets and plunged into a container with liquid propane stored in liquid nitrogen. Sample fracturing and shadowing with platinum and carbon were performed at −150 °C and in vacuum (10^{-6} Torr) on a JEE-4B vacuum station (JEOL, Japan) we had modified for freeze-fracture experiments [20]. The platinum-carbon replicas were examined on a JEM-100B transmission electron microscope (JEOL, Japan).

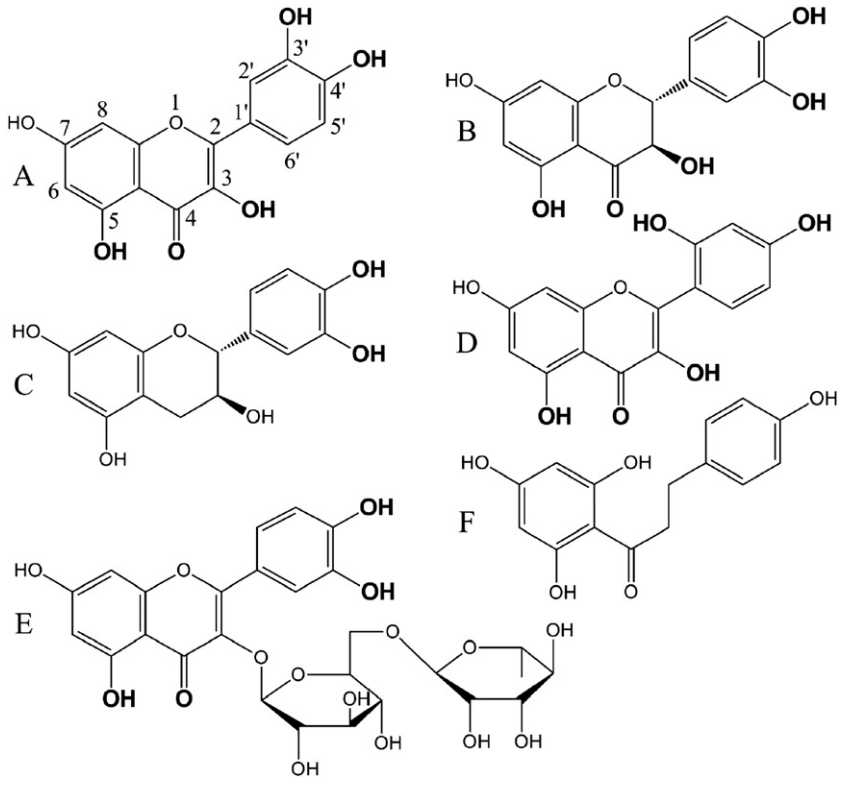

Fig. 1. Flavonoids used in our study. (A) quercetin. The numeration of carbon atoms is presented. (B) taxifolin. (C) catechin. (D) morin. (E) rutin. (F) phloretin. The moieties able to be involved in the interaction with iron are highlighted with bold letters.

2.6. Lipophilicity calculations

Lipophilicity was calculated with ChemBio3D Ultra 12.0 software package (PerkinElmer, GB) on the basis of the chemical formula of compounds. The calculated lipophilicity (CLogP) was determined as a logarithm of the partition coefficient for n-octanol/water. The calculation accuracy of this software was tested on flavonoids and a good agreement was found with the experimental data [21].

3. Results

This study was based on a visual observation of the fast increase of cloudiness in the suspension of multilamellar phospatidylcholine liposomes treated with quercetin and divalent iron. It was also found that the effect was more pronounced in the presence of calcium (data are not presented). For a more detailed and accurate investigation of the phenomenon, we used unilamellar liposomes and various flavonoids differing in the number and position of hydroxyl groups (Fig. 1).

Light scattering analysis of liposomes revealed that flavonoids themselves had no effect on the suspension, while the addition of divalent iron initiated a fast increase in light scattering of liposomal samples containing quercetin, taxifolin, catechin and morin (Fig. 2A). On the contrary, liposomal samples containing phloretin and rutin were indifferent to the addition of iron. It is notable that the rate and scale of changes varied between the samples. The amplitude of light scattering changes for samples containing taxifolin was larger than that for samples containing quercetin, catechin, and morin. The observed changes of light scattering correlated well with the concentration of flavonoid–iron complexes. An almost hundred-fold decrease of flavonoid–metal complex concentration was necessary to make the phenomenon undetectable in the scale of measurements used (Fig. 2B).

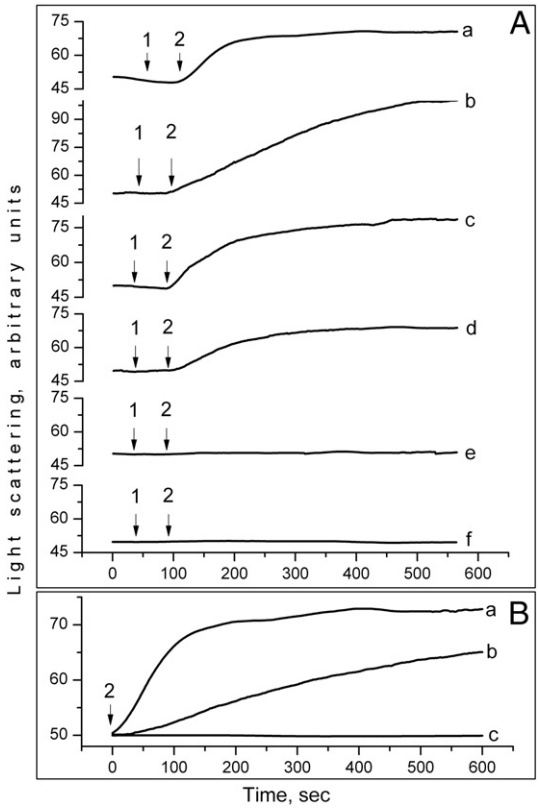

Fig. 2. Light scattering of liposomes. (A) Suspension of liposomes treated with 1×10^{-5} M flavonoid-iron complexes. The time of addition of flavonoid (1) and divalent iron (2) is indicated by arrow. Liposomes were treated with (a) quercetin, (b) taxifolin, (c) catechin, (d) morin, (e) phloretin, and (f) rutin. (B) Dependence of light scattering changes on the concentration of quercetin-iron complex. (a) The concentration was 1×10^{-5} M, (b) 1×10^{-6} M, (c) 1×10^{-7} M. Here and in the next figures (Figs. 3 and 4) the initial uprise of light scattering from about zero to about 50 relative units occurred after addition of liposomes is not given.

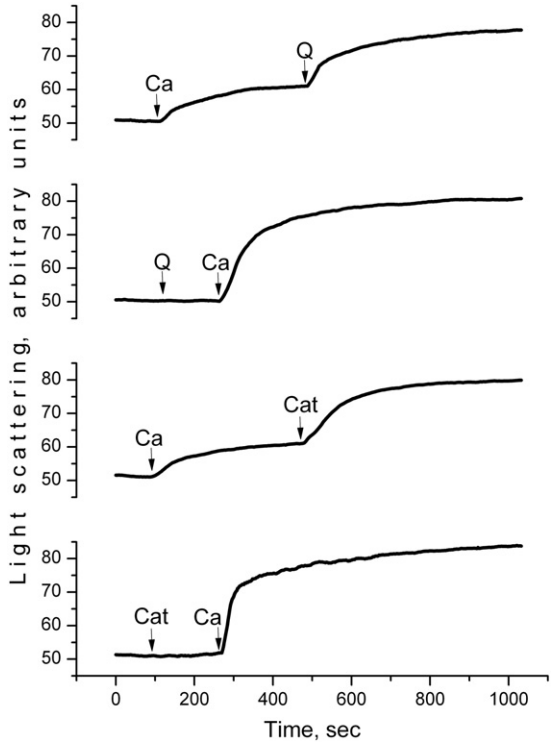

Fig. 3. Effect of calcium and flavonoids on light scattering of liposomes. Arrows indicate the moments of addition of quercetin (Q), catechin (Cat), and Ca^{2+} cations (Ca).

Light scattering of liposomes can also rise after addition of calcium (Fig. 3). When flavonoids were added following the calcium addition, two steps of light scattering increase could be seen. If flavonoids were added first, they had no effect, but the subsequent addition of calcium initiated a light scattering growth equal to or larger than the sum of two steps observed in the experiment where calcium was added first. Thus, though flavonoids alone had no influence on the light scattering, they expressed a pronounced synergetic effect when added after calcium. In experiments where calcium and iron were added to liposomes preliminary treated with flavonoids, the action of both cations was additive and did not depend on the sequence of their

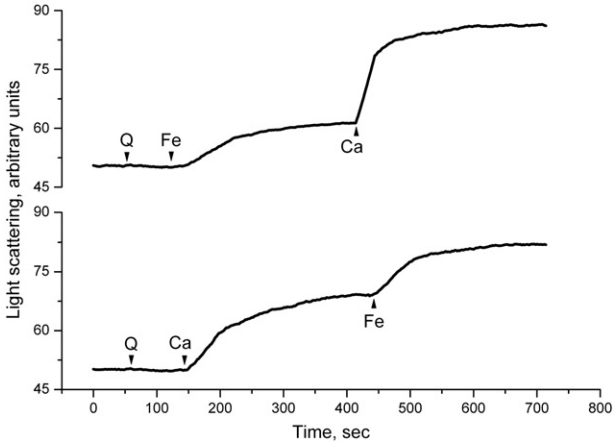

Fig. 4. Influence of quercetin (Q), divalent iron (Fe) and calcium (Ca) on light scattering of liposomes.

Table 1
Particle sizes in suspensions of liposomes treated with flavonoids, divalent iron and calcium. The data were obtained from a PCS assay (Fig. 5).

Sample	Mean size, nm	Amount, %
Liposomes	87.4 ± 25.5	100
Lip + Q + Fe	99.9 ± 35.0	100
Lip + Tax + Fe	97.9 ± 35.2	100
Lip + Cat + Fe	100.2 ± 37.6	100
Lip + Q + Fe + Ca	118.6 ± 19.1	71.94
	548.3 ± 78.5	28.06
Lip + Tax + Fe + Ca	111.7 ± 35.8	91.18
	911.3 ± 118.4	8.82
Lip + Cat + Fe + Ca	111.5 ± 23.6	83.58
	310.1 ± 48.6	16.42

addition (Fig. 4), though iron did not influence on the scattering when added before flavonoids (data not shown).

The PCS assay detected some increase in the mean size of particles after the addition of flavonoids and divalent iron to liposomes (Fig. 5 and Table 1), which correlated with the increase of light scattering observed earlier. Subsequent addition of calcium initiated formation of a new fraction with considerably larger particles. The effect was especially pronounced in the samples treated with quercetin or taxifolin.

The revealed increase in particle size can be explained by aggregation of liposomes. We expected that when liposomes aggregated, the close contacts between them might facilitate the lipid exchange and even the fusion of lipid bilayers. We found that the standard lipid exchange analysis based on FRET measurements was complicated by quenching of NBD-PE and rhodamine-PE fluorescence in the presence of flavonoids and iron. We could not estimate changes in fluorescence intensity immediately after addition of these compounds because we had to wait 10–20 min to achieve complete mixing of compounds and the equilibration of samples. It was found that the quenching effect, though resulting in a nearly 30% decrease of the fluorescence intensity, did not introduce significant changes both in the shape of spectra and in the position of maximums. According to the light scattering and PCS experiments presented above, the most considerable changes of parameters occurred after addition of calcium to liposomes pretreated with flavonoids and iron. Therefore we started the registration of fluorescence intensity after the addition of calcium. It was found that the fluorescence of NBD-PE ($\lambda = 537$ nm) slowly increased (Fig. 6) while the fluorescence of rhodamine-PE ($\lambda = 590$ nm) decreased (Fig. 6), which demonstrated a decrease in FRET between dye molecules. The decrease in FRET can be explained by the increase of distance between fluorescent moieties, which is caused by lipid exchange between stained and unstained liposomes and dilution of fluorescing molecules in the bilayer. The half-time of changes observed in FRET assays was in the range from 30 to 60 min.

Freeze-fracture electron microscopy of liposomes (Fig. 7) demonstrated that the original size of vesicles was between 50 and 150 nm. In samples frozen within 30–40 min after addition of flavonoids, divalent iron, and calcium, one could recognize the aggregation of some liposomes and the formation of contacts between them, though a considerable number of liposomes did not aggregate. Some samples also contained gigantic liposomes surrounded by adhered small

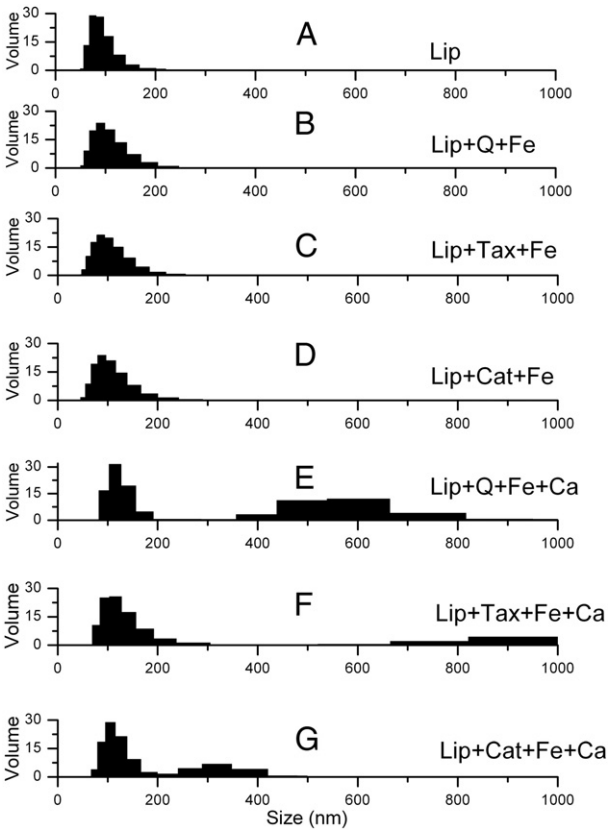

Fig. 5. PCS analyzes of liposomes (A) Before treatment. (B–D) After addition of quercetin (Q), taxifolin (Tax) and catechin (Cat). (E–G) After addition of Ca^{2+} to liposomes preliminary treated with flavonoids and divalent iron.

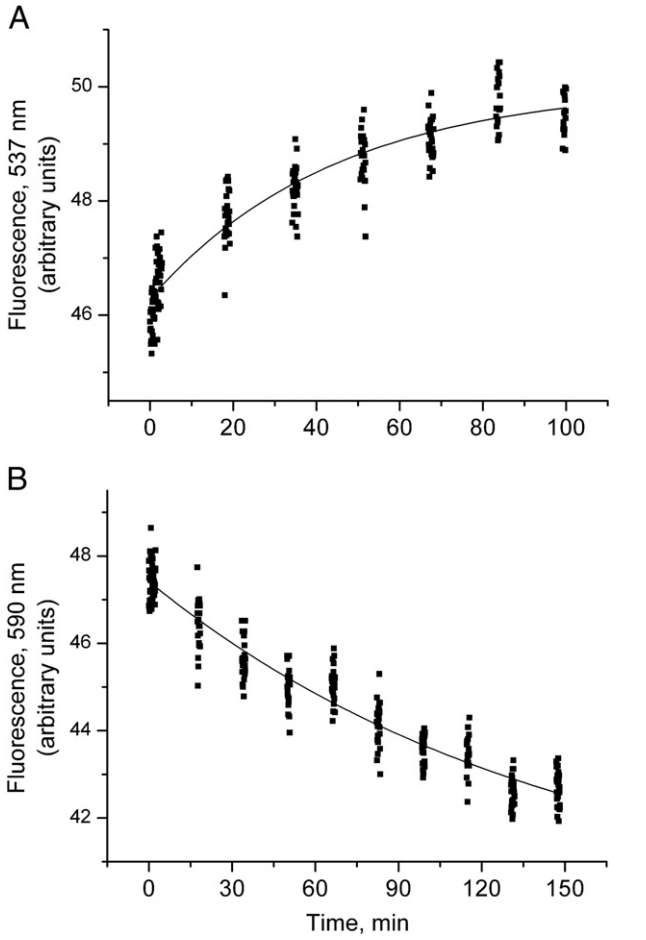

Fig. 6. Fluorescence of liposomes containing NBD-PE and rhodamine-PE dyes. The excitation $\lambda = 450$ nm. Fluorescence was registered at $\lambda = 537$ nm (A) and $\lambda = 590$ nm (B) in two independent experiments. The recordings started after addition of calcium to liposomes treated by quercetin and iron and equilibrated for about 20 min at continuous stirring. In the experiments presented, the short periods of recordings were alternated by 15 min pauses. The solid exponential curves represent the computer approximation of experimental data. The presented changes in fluorescence intensity range from 30 to 50% of the total changes observed after addition of Triton X100 and complete dilution of fluorescent probes (not shown).

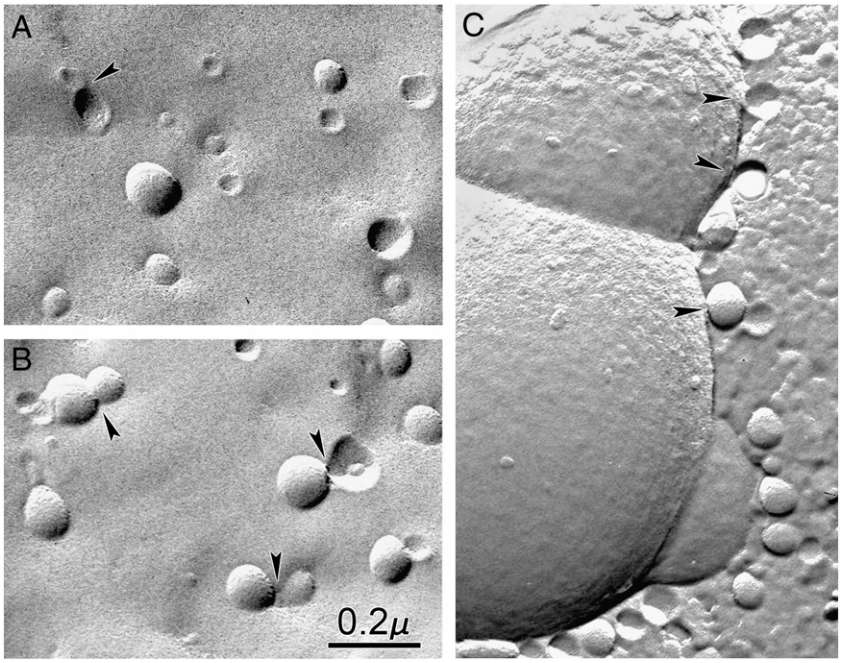

Fig. 7. Freeze-fracture electron microscopy of liposomes before (A) and after (B, C) addition of quercetin, divalent iron and calcium. The contacts between liposomes are indicated by arrow heads.

liposomes. The observed heterogeneity in behavior and sizes of liposomes correlates with the data of PCS assay presented above and could be explained by an insufficient rate of mixing of the components, which may lead to an uncontrolled increase in local concentrations of additives. The formation of gigantic liposomes in suspension can be explained only by fusion of small liposomes, which is induced by addition of flavonoids, iron, and calcium. Thus, the electron microscopy data also correlate with the above FRET measurements of lipid exchange between liposomes.

4. Discussion

The ability of PC liposomes to form aggregates, exchange lipid and to fuse was extensively investigated. It was found that fusion abilities

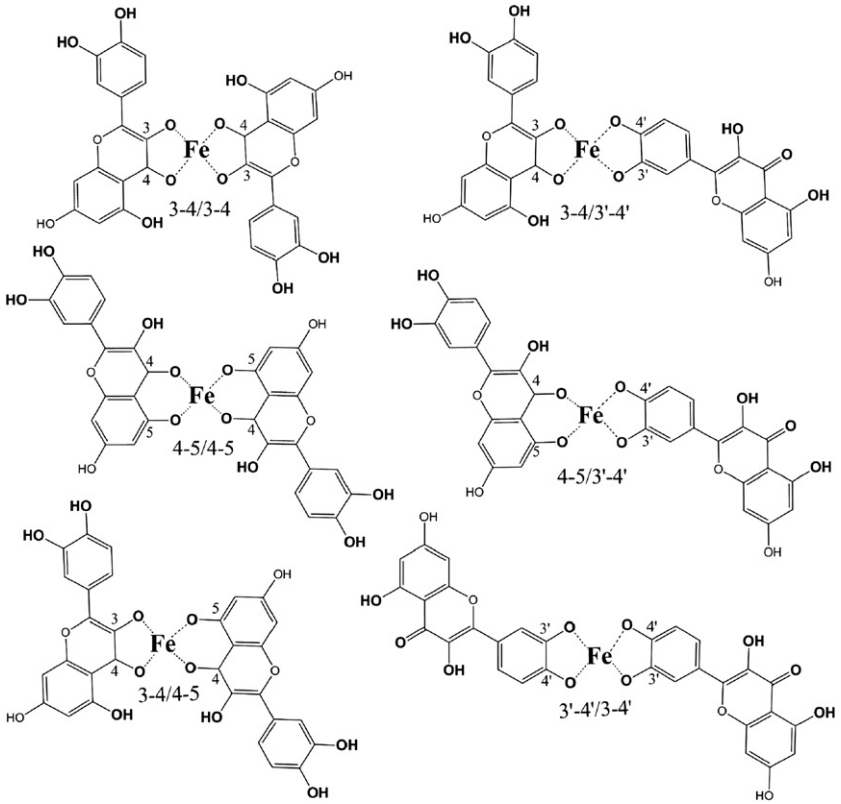

Fig. 8. Possible variations of complexes between quercetin and divalent iron. The presented numerical designation of complexes correlates with the carbon atom numeration given above (Fig. 1). Here only cis-isomers are presented.

Table 2
Calculated values of lipophilicity (cLogP) for complexes of flavonoids and divalent iron. Cis- and trans-isomers of complexes are not presented because their parameters were identical. The most lipophilic complexes are highlighted by the bold font.

Complex	Quercetin	Taxifolin	Catechin	Morin
Free compound	1.504	0.771	0.534	1.134
3-4/3-4	3.478	1.966	–	3.338
4-5/4-5	**3.961**	1.401	–	**3.821**
3-4/4-5	3.719	1.774	–	3.579
3-4/3′-4′	3.572	2.087	–	–
4-5/3′-4′	3.814	1.851	–	–
3′-4′/3′-4′	**4.063**	**2.605**	**2.131**	–

of PC liposomes depend on their size and physical state of lipid bilayer. Thus, PC liposomes less than 100 nm can spontaneously aggregate and fuse at temperatures above the lipid melting point, though the process takes many hours and days [22]. The polyvalent metal cations like calcium or uranyl can initiate aggregation of large PC liposomes [23,24], though the fusion was not detected. Thus, it has been agreed that PC liposomes equal or larger than 100 nm are essentially non-fusogenic. A pronounced aggregation and fusion of small and constrained PC liposomes could be initiated by pH changes [25,26]. Different compounds, like ethanol [27], free fatty acids [28], polyethylene glycol [29,30], fusogenic peptides [31], clathrin [32], tubulin in the presence of calcium [33], and even complexes of DNA with divalent metal cations [34] can initiate the fusion of small PC liposomes. Calcium can initiate the fusion of liposomes composed by the mixture of PC and negatively charged lipids, like phosphatidylserine [35–37], phosphatidic acid [38,39], or cardiolipin [40,41]. Generally, the rate of liposomal fusion declined when the content of PC in the mixture was elevated. This finding was defined as the inhibitory effect of PC on membrane fusion [42,43].

In this study we presented evidence for PC liposome aggregation, lipid exchange and liposome fusion initiated by flavonoid-iron complexes and calcium. Some flavonoids are known to bind cations of divalent iron in the sites of adjacent enol- or keto-enol-groups. Quercetin has three sites of binding in the positions 3–4, 4–5 and 3′–4′. The divalent iron can bind two molecules of quercetin [44]. This implies that at least six variations of quercetin-iron complexes are possible (Fig. 8). Each variation may have cis- and trans-isomers.

The calculated value of lipophilicity (cLogP) can be used to estimate the ability of flavonoid-iron complexes to interact with the lipid bilayer. According to the calculations, cLogP may vary considerably between the complexes. In all instances under consideration, the complexes of flavonoids with iron were more lipophilic than corresponding free flavonoids (Table 2). For quercetin complexes with divalent iron, the variations 3′-4′/3′-4′ and 4-5/4-5 were most lipophilic ones. The lipophilicity of complexes of taxifolin with iron was much lower, but in this case the 3′-4′/3′-4′ complex was also the most lipophilic one. Morin and catechin had fewer variations of complexes, but again 3′-4′/3′-4′ was the most lipophilic complex of catechin, and 4-5/4-5 was the most lipophilic complex of morin. Phloretin is unable to form complexes with iron, while rutin cannot interact with the lipid bilayer [45]. Accordingly, we can suppose that both compounds would unable to initiate liposomal aggregation which is confirmed by our data presented above.

The number and distribution of hydroxyl groups in flavonoid molecules may determine their interaction with the lipid bilayer [46]. In complexes of flavonoids with iron, the oxygen charges are generally decreased while the main negative charge is localized on the iron atom (Fig. 9). According to calculations presented, after helating the iron, the polarity of flavonoid molecules generally decreased, while the iron atom becomes the most polar and hence hydrophilic part of the molecule. This implies that the iron can serve as a polar link between two flavonoid molecules anchored hydrophobically to the adjacent bilayers. We suppose that flavonoid molecules are able to hold together the neighboring bilayers through the links produced by iron thus initiating the aggregation of liposomes. It is possible that different types of complexes can be involved in liposome aggregation but the most lipophilic complexes may reveal the highest effectiveness. On the scheme (Fig. 10), we present some examples of interbilayer links produced by iron and flavonoids.

We also found the additivity in the action of divalent iron and calcium on the liposomal aggregation (Fig. 4). However, iron appeared much more efficient than calcium, and to achieve similar effects, we used a 10-times higher concentration of calcium than that of flavonoid-iron. It is known that calcium cations can produce bridges between adjacent bilayers, decrease the repulsion forces between them, and facilitate their interaction and fusion [47]. Indeed, the addition of calcium to liposomes containing flavonoids and iron facilitated the aggregation and initiated liposome fusion, which was recorded by FRET spectroscopy (Fig. 6) and electron microscopy (Fig. 7).

The ability of polyphenolic compounds, like tannins, to initiate membrane adhesion is well known [48,49]. It was found that tannins can interact with two adjacent bilayers and produce bridges between them. This mechanism is effective because tannin molecules are large enough to penetrate into the hydrophobic regions of neighboring membranes. Molecules of flavonoids are considerably smaller than those of tannins, so that each flavonoid molecule can interact only with one bilayer. They cannot form bridges between bilayers thus facilitating membrane adhesion. However, it is known that in the presence of some transient metals like iron, flavonoids can form dimers [14]. According to our calculations the lipophilicity of flavonoid-iron complexes was considerably higher than that of free flavonoids. Hence, complexes of flavonoids with iron can interact with two bilayers and connect them through the atom of iron acting as a link.

The possible involvement of polyphenols in the processes of membrane adhesion is a very important aspect of their functioning in

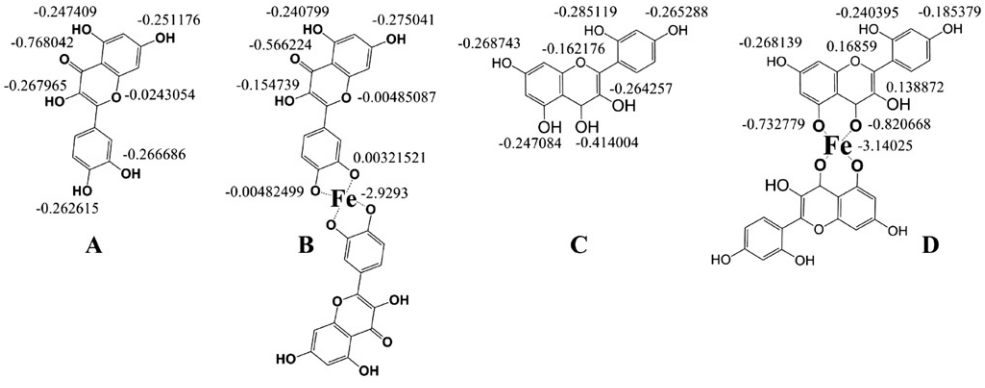

Fig. 9. Huckel charges calculated for oxygen and iron atoms in molecules of flavonoids and their complexes with iron. (A) Quercetin. (B) Quercetin-iron complex (3′-4′/3′-4′). (C) Morin. (D) Morin-iron complex (4-5/4-5).

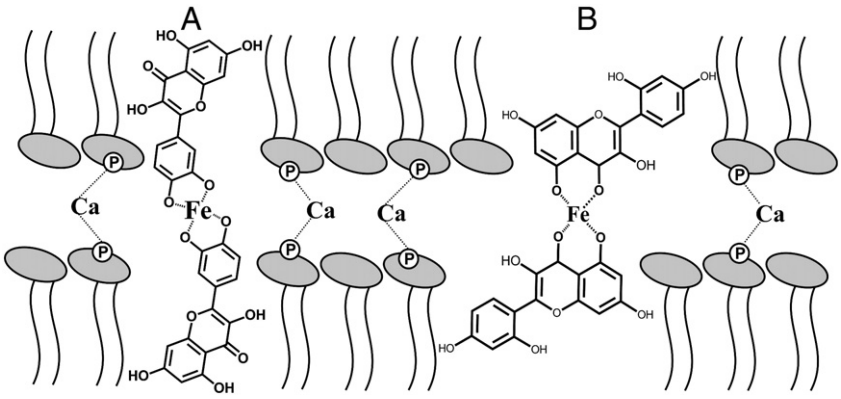

Fig. 10. Model of iron links between two adjacent liposomes formed by. (A) Quercetin-iron complex 3′-4′/3′-4′. (B) Morin-iron complex 4-5/4-5. Calcium bridges between phosphates are also presented.

living cells and needs further study [50]. The supposed increase in adhesion between cellular membranes, under the action of polyphenolic compounds or their complexes with transient metals, may influence endo- and exocytosis, which could help us to explain the protective effect of polyphenols on cells against invasion by infective factors [4,51,52], or inflammatory processes [53]. Indeed, as it was found, tannic acid can retard the formation of membrane vesicles during endo- and exocytosis [54,55]. Quercetin can influence not only membrane adhesion but also the expression of adhesion proteins responsible for endocytosis and the transport of compounds into the cytoplasm [52]. Studies on involvement of polyphenol in interaction between membranes may also help to elucidate the mechanisms of the cell-cell communication or microflora colonization in the human body [56,57].

Acknowledgements

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