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The effect of *trans*-resveratrol on the physicochemical properties of lipid membranes with different cholesterol content

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ABSTRACT

Resveratrol is one of the most popular phytoalexins, which naturally occurs in grapes and red wine. This compound not only has beneficial effects on the human body, especially on the cardiovascular system, but also has antiviral, antibacterial and antifungal properties. In addition, resveratrol may have therapeutic effects against various types of cancer. The mechanism of action of resveratrol is not fully understood, but it is suspected that one of the most important steps is its interaction with the cell membrane and changing its molecular organization. Therefore, in the present study, we investigated the effects of resveratrol at different concentrations (0–75 μM) on model membranes composed of POPC, SM and cholesterol, in systems with different cholesterol contents and a constant POPC/SM molar ratio (1:1). Our tests included systems containing 5, 15 and 33.3 mol% cholesterol. Tests were carried out for monolayers using the Langmuir monolayer technique supported by Brewster angle microscopy and penetration experiments. Bilayer (liposome) experiments included calcein release, steady-state DPH fluorescence anisotropy and partition coefficients. The results showed that resveratrol interacts with model cell membranes (lipid monolayers and lipid bilayers), and its incorporation into membranes is accompanied by changes in their physicochemical parameters, such as lipid packing, fluidity and permeability. Furthermore, we showed that the cholesterol content of the membrane significantly affects the degree of incorporation of resveratrol into the model membrane, which may indicate that the molecular mechanism of action of this compound is closely related to its interactions with lipid rafts, domains responsible for regulating various cellular functions.

1. Introduction

Resveratrol belongs to the group of phytoalexins, which are found mainly in grapes and red wine [1,2]. However, it can also be found in other plants, such as peanuts, coco, cranberries,

blueberries, and soybeans [3–5]. Resveratrol can occur in the form of two isomers, *cis* and *trans*, but the *trans* form shows the greatest biological activity [6], while the *cis* isomer is considered much less bioactive or completely inactive [7,8]. The main function of the resveratrol present in plants is to protect them against environmental stressors, such as injuries, fungal infections, or UV irradiation [9,10]. However, resveratrol was also observed to have a beneficial effect on the human body, especially its antioxidant and preventive effects on the cardiovascular system [11]. A good example of the beneficial effects of resveratrol on the human body is the phenomenon called the "French paradox". The French population, compared to Western European countries, consumes much larger amounts of animal fats, and yet the incidence of atherosclerosis in France is much lower. The relatively high level of consumption of red wine, which is rich in resveratrol, is assumed to be an explanation for this phenomenon [6,12,13]. The above explanation is of course a great simplification, because the French diet does not consist only of fatty meats, cheeses, cream and red wine, but also contains many low-processed products, vegetables, and fruits. In addition, the resveratrol content of wine is rather low, and can range from undetectable values of up to 14 mg/L [14,15], which is well below its therapeutic dose, which according to some meta-analyses should be above 300 mg/day [16]. Regardless of whether the French paradox really exists or is actually related to resveratrol, it has contributed to broader research on the effects of resveratrol on the human body. These studies have shown that resveratrol can also regulate the serum glucose level, as well as have antimicrobial, antifungal, antiviral, anti-inflammatory and neuroprotective properties [4,17,18]. Recent

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reports also indicate that resveratrol has antiviral properties and may also be a helpful antiviral agent in the treatment of viral infections such as COVID-19 [19]. In addition, one of the most significant health benefits of resveratrol is its ability to produce chemopreventive and therapeutic effects against various cancers [18]. These properties were first discovered in 1997 by Jang et al., who demonstrated the anti-initiation, anti-promotion, and anti-progression effects in various models [20]. Based on these studies, other scientists have shown that resveratrol inhibits tumor growth in vivo in several types of cancer, with effects dependent on dose and duration of treatment [18,21,22]. It is worth mentioning here, however, that there is an ongoing debate among scientists regarding the limitations of resveratrol, its therapeutic dose and its effectiveness [14,23–25]. Nevertheless, as the authors of the meta-analysis undermining the effectiveness of resveratrol themselves note, they have serious limitations and further research on the effect of this substance on the human body is required [24].

Although resveratrol may have a huge therapeutic potential, its use in treatment is very limited. This is primarily related to its bioavailability and its targeting of specific sites in the body, particularly the brain. The poor bioavailability of resveratrol may be due to its short biological half-life, chemical lability, rapid metabolism, enterohepatic recirculation and elimination [3,25–28]. The short half-life and rapid metabolism of resveratrol require a higher dose and frequent administration to achieve a therapeutic effect. However, resveratrol is cytotoxic at the higher total doses required to achieve a relatively high local concentration and thus a therapeutic effect [3,29]. Furthermore, resveratrol is photosensitive, poorly soluble in water, and poorly absorbed after oral administration [3]. To overcome the problems associated with the chemical instability and rapid metabolism of resveratrol and its bioavailability, more and more research is being carried out to design new formulations that stabilize and protect resveratrol, improve its water solubility, and provide targeted and/or sustained release as well as allow its intravenous administration. So far, complexes with β-cyclodextrins, biodegradable polymer nanoparticles, solid lipid nanoparticles and polymer lipid nanocapsules have been studied. Among the formulations tested, liposomes containing resveratrol were also found to be effective in cell proliferation, photoprotection, and cellular stress response [3,28,29].

The exact mechanism of action of resveratrol is not fully understood and is probably very complex and multi-stage. However, the first platform with which resveratrol interacts is the cell membrane. The biomembrane is also the main barrier that resveratrol molecules must overcome on the way to a specific place in the cell. Previous studies have shown that resveratrol has the ability to incorporate into biomembranes, changing their physicochemical properties [1,7,8], which, depending on the dose, can even lead to cell lysis [30].

The ability of resveratrol to accumulate in biological membranes as well as the use of liposomes as effective resveratrol nanocarriers require systematic research on the effect of this substance on the physicochemical properties of lipid membranes. Therefore, in our study, we attempted to determine the effect of the concentration of resveratrol on the properties of lipid membranes of different composition, and we also examined how the affinity of resveratrol for lipid membranes is determined by their physicochemical parameters. In the research, we used two models of biomembranes, i.e. lipid monolayers and bilayers with the same composition. The studies included monolayers and bilayers (liposomes) containing lipids that are components of biological membranes, i.e. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), sphingomyelin (SM) and cholesterol (Chol). The tested systems contained the same proportion of phospholipids (POPC/SM) and differed in cholesterol content (5, 15, 33.3 mol%). This allowed us to determine the effect of resveratrol concentration on a particular membrane, as well as to compare how the membrane properties determined by the cholesterol content affect the incorporation capacity of resveratrol. Furthermore, the investigated lipid membranes contained SM and Chol, which allowed us to verify the effect of resveratrol on model lipid rafts, that is, highly ordered domains enriched in sphingomyelin and cholesterol, the number of which increases rapidly in the carcinogenesis process [31].

2. Materials

The investigated materials POPC (1palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), SM (egg sphingomyelin), Chol (cholesterol), Res (resveratrol). Sterol was purchased from Sigma, while the other lipids were from Avanti Polar Lipids, Inc. Resveratrol was purchased from TCI Tokyo Chemical Industry, Japan. The other compounds used in the experiments: bis[N,N–bis(carboxymethyl)aminomethyl]fluorescein (calcein) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma Aldrich. PBS buffer solution was prepared by mixing the appropriate amounts of inorganic salts (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4) and dissolving in ultra-pure water, HPLC – grade (>99,9 %). Methanol and chloroform were purchased from POCH and used as a mixed solvent (1:9 *v*/v) for dissolving of the lipids. The mixed solutions were prepared by mixing the proper volumes of the respective stock solutions. A stock solution of resveratrol was prepared in PBS buffer at a concentration of 150 μM. The method of preparing the solution consisted of weighing the appropriate amount of resveratrol adding it to the PBS buffer and stirring in the dark for 12 h at 250 rpm at 37 °C.

3. Methods

3.1. Langmuir monolayer technique

The stock solutions of the investigated lipids of concentration 0.8–1.0 mM were prepared by dissolving the respective compound in a mixture of chloroform/methanol 9:1 (*v*/v); whereas, the mixed systems of a specified composition were prepared from the stock solutions by mixing their appropriate volumes. Surface pressure (π) - Area (A) isotherms were recorded with the NIMA (UK) Langmuir trough of total area of 300 cm^2 placed on antivibration table. Surface pressure measurements were made using Wilhelmy plate made of filter paper (ashless Whatman Chr1) connected to an electrobalance with an accuracy of ±0.1 mN/m. As a subphase pure PBS buffer (pH = 7.4) and buffered resveratrol solutions (5, 15, 25, 50, 75 μM) were applied. The temperature of the subphase (20 ± 1 °C) was controlled thermostatically. The study was conducted at 20 °C because this temperature, unlike physiological temperature (37 °C), ensures the recording of high-quality images of monolayers using Brewster angle microscopy (BAM). In addition, a temperature of 20 °C, like 37 °C, is above the phase transition temperature for the POPC membrane and below the phase transition temperature for the SM bilayer. This means that taking measurements at a higher temperature could change the results quantitatively but not qualitatively. The monolayers tested were prepared by spreading, using a Hamilton microsyringe (precise to ±1 μL), an appropriate volume of solution on the subphase. Before compression, the monolayers were allowed to equilibrate for 30 min, which was the time required for resveratrol to be incorporated into the monolayer. This time was selected experimentally and its extension did not affect the shape and location of the recorded isotherms. Since no influence of compression velocity (within the range of 1–6 $Å^2 \cdot molecule^{-1} \cdot min^{-1}$) was found for the investigated films, in all experiments the monolayers were compressed with a speed of 3.5 $Å^2 \cdot molecule^{-1} \cdot min^{-1}$. The measurements were conducted for POPC, SM, Chol monolayers as well as POPC/SM, POPC/SM/Chol(5 %), POPC/SM/Chol(15 %) and POPC/SM/Chol(33.3 %) systems in which the molar ratio of POPC/SM was equal 1:1.

3.2. Brewster angle microscopy

Brewster angle microscopy was used as a technique for examination of the morphology of the analyzed monolayers. This technique allows in situ observations of changes in the monolayer texture. The intensity of

reflected light from the monolayer is adequate for the packing density molecules in the monolayer. This method also enables the observation of phase separation, and for homogeneous monolayers, it enables the comparison of their condensation based on differences in image brightness. BAM experiments were performed with an UltraBAM instrument (Accurion GmbH, Göttingen, Germany) equipped with a 50 mW laser emitting light of p polarization at a wavelength of 658 nm, a 10× magnification objective, a polarizer, an analyzer, and a CCD camera. The spatial resolution of the BAM was 2 μm. The microscope was installed over the KSV Langmuir trough (total area of 700 cm^2) having two moving barriers enabling symmetrical compression. The preparation of Langmuir films for measurement and the conditions during measurements were the same as those applied to the π-A isotherms registration.

3.3. Preparation of unloaded liposomes

Liposome formulations were obtained by the dry lipid film method by mixing the appropriate amounts of stock lipid solutions. Then, the solvents were evaporated under a gentle stream of nitrogen until complete dryness. The obtained dry lipid film was hydrated in PBS (phosphate buffered saline, pH = 7.4) and vortexed. The final concentration of lipids in all systems was 1.0 μmol/ml. To obtain unilamellar liposomes, suspensions were subjeted to five cycles of freezing (liquid nitrogen temperature) and thawing (temperature around 60 °C). Finally, the prepared mixture of liposomes was extruded six times through the polycarbonate filters with 100 nm pores with the appliction of LiposoFast extruder (Avestin Inc.).

3.4. Preparation of calcein-loaded liposomes

Calcein-loaded liposomes were performed in the same procedure as described above. The dry film was hydrated with calcein solution (60 mM calcein dissolved in 0.1 M PBS at pH = 7.4). In the next step, free calcein molecules were separated by size exclusion chromatography on a sephadex G-50 column using 0.1 M PBS buffer as an eluent (the PBS was enriched by increasing the concentration to 60 mM NaCl to normalize the concentration between enclosed calcein and the outer environment). The final concentration of liposomes after the separation of calcein-loaded liposomes from free dye was calculated according to the procedure described in the literature [32].

3.5. Preparation of DPH liposomes and anisotropy measurements

To prepare the liposomes with the fluorescence probe inside the membrane, 1,6-Diphenyl- 1,3,5-hexatriene (DPH) was added to the propper volume of the lipids. The DPH concentration in the liposome membrane was 1.67 μM. The subsequent procedure was similar to that described for unloaded liposomes. The fluorescence anisotropy was determined using vertically polarized light at λ = 350 nm for excitation and parallel (I_{VV}) and perpendicular (I_{VH}) light at λ = 428 nm for emission. In these measurements Hitachi 7100 spectrofluorometer was used. All measurements were repeated three times and after that the average value was calculated. The steady - state anisotropy (r_{DPH}) was calculated according to the Eq.:

$$r_{DPH} = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

where: $G = I_{HV}/I_{HH}$ instrumental correction factor. The measurements were carried out at 20 °C.

3.6. Partition coefficient

To determine the partition coefficient for all the liposome compositions investigated, samples of different total lipid concentrations were prepared in the range 0-1000 μM. The concentration of resveratrol was constant (25 μM). Liposomes without resveratrol addition were prepared and measured as a background. Liposomes with resveratrol were incubated for 30 mins after resveratrol addition. The samples were measured using UV–vis spectrometry and the partition coefficient was determined with procedure described in the literature [33,34]. The measurements were carried out at 20 °C.

3.7. Permeability studies

The permeability of liposomes was determined at 20 °C by measuring the dispersions of calcein-loaded liposomes for 14 days using fluorescent detection. After 14 days, Triton X-100 was used to completely break down the vesicles and measure the maximum fluorescence. The amount of calcein released was calculated using an equation based on the literature [32].

$$RT\% = \frac{I_t - I_0}{I_{max} - I_0} * 100\%$$

where: I_t – fluorescence intensity in time t, I_0 – fluorescence in the beginning, I_{max} – maximum fluorescence intensity after Triton X-100 addition.

4. Results

4.1. Langmuir monolayers

In the first step, π-A isotherms for one-component monolayers of the investigated lipids, compressed on the water subphase, were recorded. The results obtained were consistent with those described in the literature [35]. Then we recorded the π-A isotherms for monolayers of pure lipids SM, POPC, Chol as well as their mixtures (POPC/SM, POPC/SM/Chol(5 %), POPC/SM/Chol(15 %), POPC/SM/Chol(33.3 %)) spread on the PBS subphase and the PBS subphase containing resveratrol with different concentration i.e. 5 μM, 15 μM, 25 μM, 50 μM, 75 μM. The results obtained for mixed monolayers are shown in Fig. 1, while the isotherms for pure lipid films are included in the supplementary materials (Fig.S1). Moreover, to obtain information about the state and ordering of hydrophobic chains of the analyzed monolayers, the compressional modulus were calculated for all the investigated systems according to the equation:

$$C_s^{-1} = -A\left(\frac{d\pi}{dA}\right)$$

As can be seen, for all the investigated systems, an increase in the concentration of resveratrol in the subphase causes the isotherms to shift toward larger areas per molecule. Additionally, as the concentration of resveratrol increases, the compressional modulus decreases. Both of the above effects are stronger with higher concentration of resveratrol in the subphase. This suggests that resveratrol dissolved in the subphase has the ability to build into the lipid monolayer and changes its molecular organization, resulting in an increase in its fluidity. It can also be observed that the effect of resveratrol on the investigated monolayers strongly depends on the cholesterol content in the model membrane. The results obtained clearly indicate that the isotherm shift caused by the presence of resveratrol, in relation to the isotherm recorded on pure PBS, is the smaller the more cholesterol is contained in the mixed monolayer. This is probably due to the condensing and ordering effect that cholesterol exerts on lipid membranes [36,37]. These effects cause monolayers containing cholesterol to be more densely packed and ordered than those without cholesterol. This is manifested by the location of the isotherms at smaller average molecular areas, their steeper course, and higher values of the compression modulus with increasing cholesterol content.

In order to obtain information on the influence of resveratrol on the

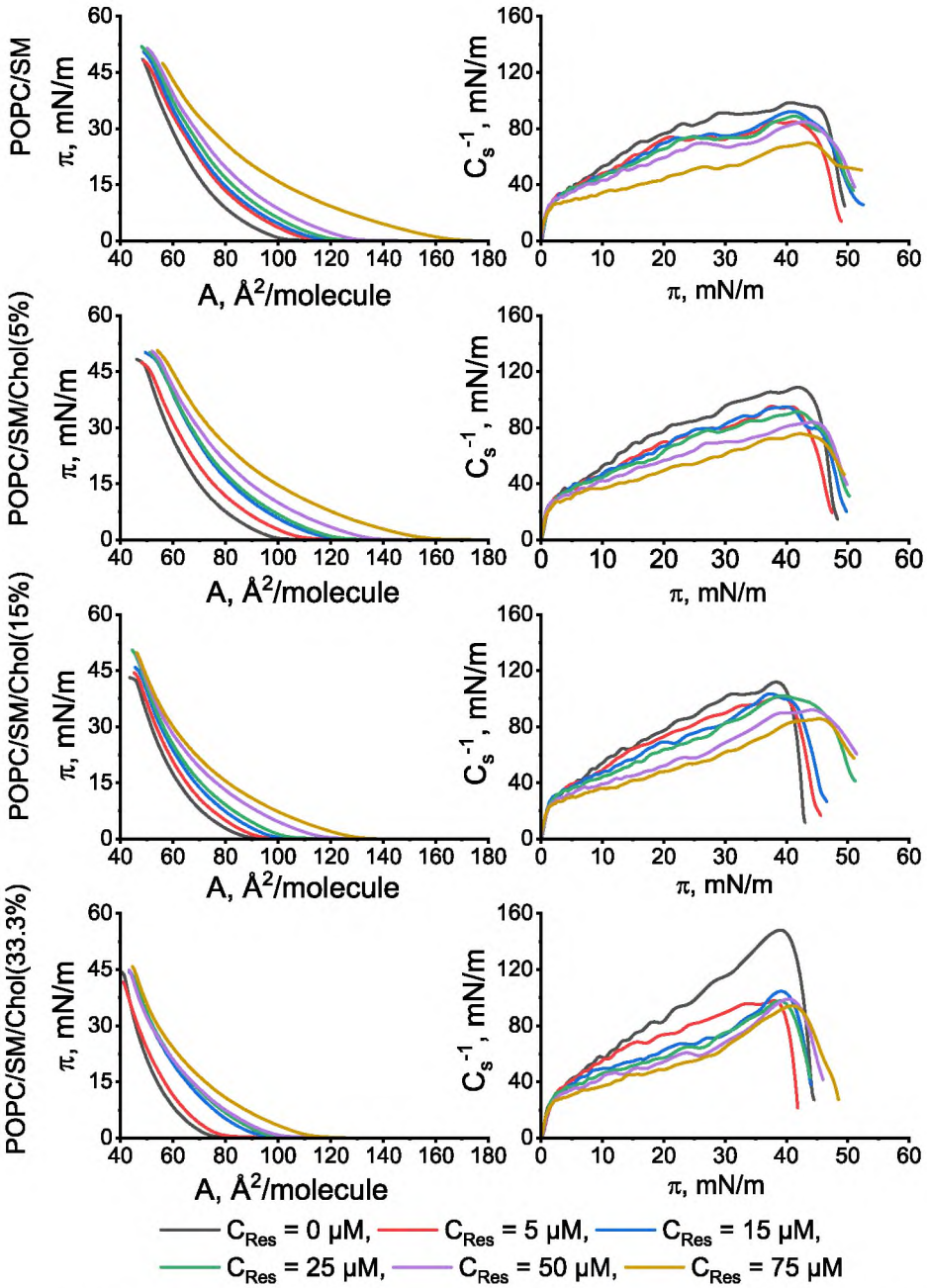

Fig. 1. The surface pressure (π) – area (A) isotherms and compression modulus (C_S^{-1}) vs. surface pressure plots for the mixed systems investigated.

organization and morphology of lipid membranes, BAM images were taken during compression of selected membranes. In the first stage, the influence of the concentration of resveratrol in the subphase on the morphology of the mixed POPC/SM/Chol monolayer containing the same amount of cholesterol (15 mol%) was investigated. Pictures were recorded for monolayers spread on pure PBS subphase as well as for PBS subphase containing resveratrol in the concentration of 5 μM, 15 μM and 50 μM, respectively. The images obtained are presented in Fig. 2.

For the monolayer compressed on the pure PBS subphase, in the low surface pressure range (0.3 and 5 mN/m), the photos show brighter domains of the condensed phase dispersed in the darker matrix of the liquid phase. These condensed domains are probably enriched in sphingomyelin and cholesterol, and the liquid matrix consists mainly of POPC. As this monolayer is compressed to higher surface pressures, the domains gradually fuse together to form a homogeneous film of the liquid-condensed phase until the monolayer collapses. The introduction of a small amount of resveratrol (5 μM) to the PBS subphase causes that the condensed phase domains that are formed to be much smaller, practically at the limit of the microscope's resolving power. A further increase in the concentration of resveratrol in the subphase causes the condensed phase domains to not completely form, which confirms the fluidizing effect of resveratrol on the investigated monolayers. It should also be noted that the BAM images become brighter as the resveratrol content in the subphase increases. This confirms that as the concentration of resveratrol in the water phase increases, more and more resveratrol molecules are incorporated into the model membrane. It is worth noting, however, that the increase in the brightness of the images does not mean, in this case, an increase in the order of the lipid acyl chains, but is related to the increasing number of molecules at the air/water interface as a result of the increasing incorporation of resveratrol into the membrane.

In the next stage, we examined how resveratrol influences the

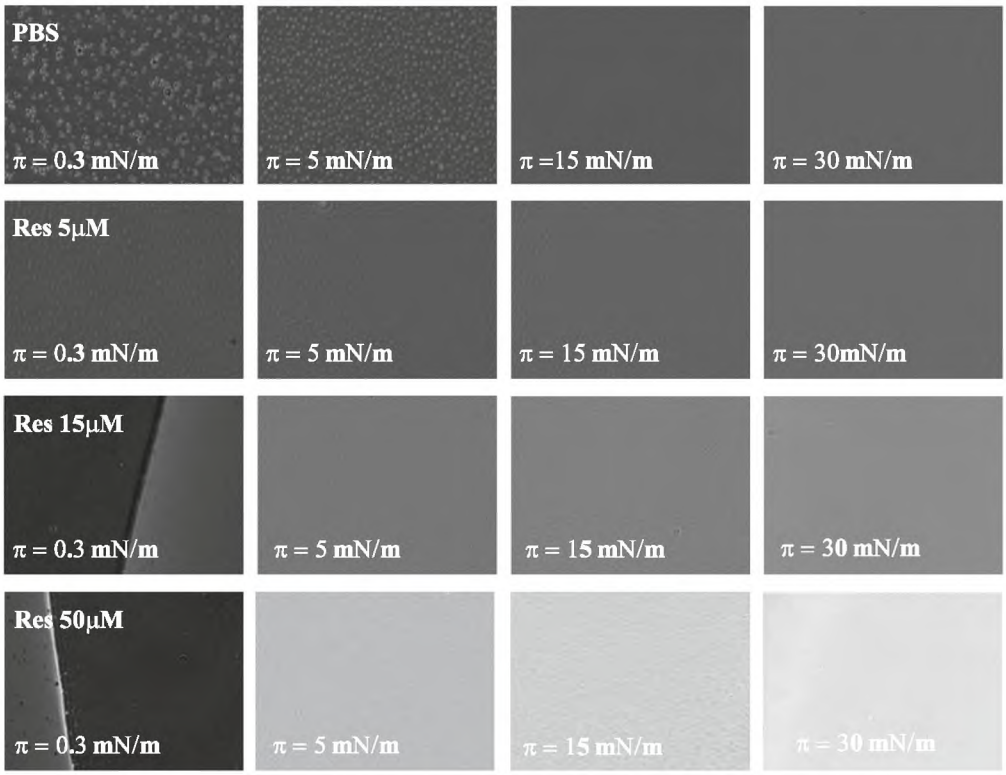

Fig. 2. BAM images obtained for the POPC/SM/Chol(15 %) system spread on the subphases containing different concentrations of resveratrol.

morphology of monolayers depending on the cholesterol content. For this purpose, pictures were taken for POPC/SM/Chol monolayers with different cholesterol content (0, 5, 15, 33.3 mol%) during compression onto the PBS subphase containing 15 μM resveratrol. The images obtained are presented in Fig. 3.

The analysis of the results presented in Fig. 3 shows that the main

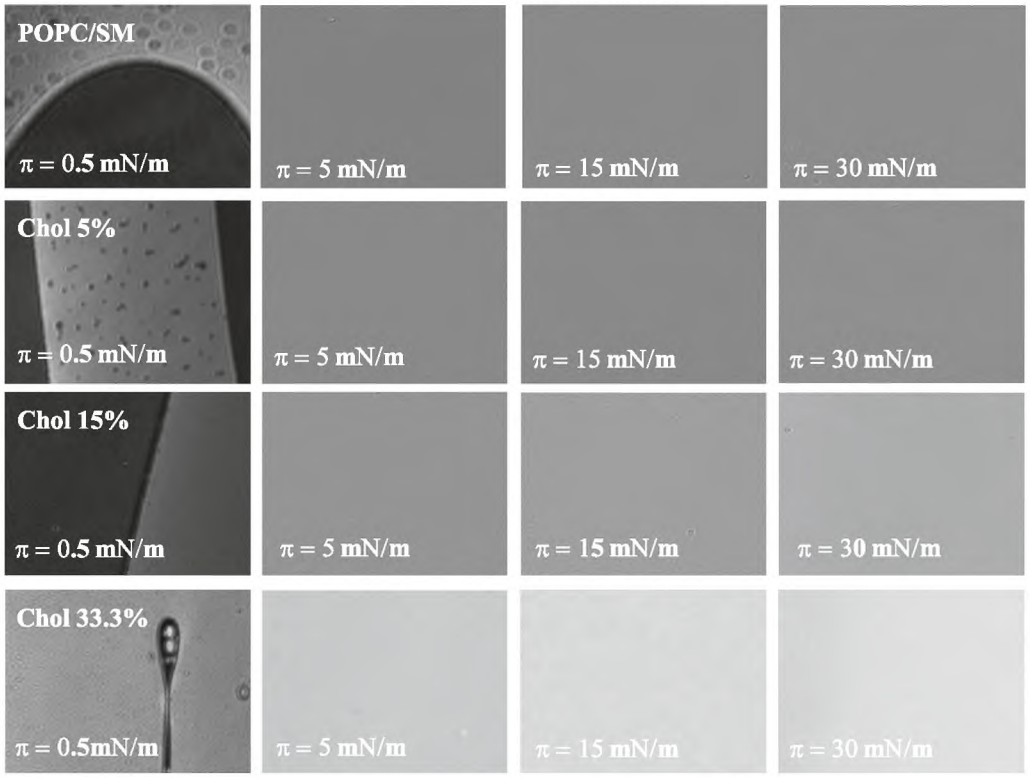

Fig. 3. BAM images obtained for POPC/SM/Chol monolayers with different cholesterol content (0, 5, 15, 33.3 mol%) during compression onto the PBS subphase containing 15 μM resveratrol.

difference is the degree of condensation of the investigated monolayers observed in the range of low surface pressures and a slight increase in the degree of brightness of the images as the cholesterol content in the monolayer increases. The differences in the brightness of the images are not spectacular because we are dealing with two opposing effects, the first is related to the condensing effect of cholesterol on phospholipid membranes and the second is the result of the decreasing incorporation of resveratrol into the model membrane as the cholesterol content increases.

To confirm the ability of resveratrol to be incorporated into model lipid membranes, as well as to determine the effect of cholesterol content in the membrane on this process, penetration tests were performed. These tests were carried out for mixed POPC/SM/Chol monolayers containing, respectively, 0, 5, 15 and 33.3 mol% cholesterol at a surface pressure of 30 mN/m, i.e., at a pressure for which the packing of lipids and their molecular organization correspond to that in the lipid bilayer with the same composition. The results of the penetration studies of resveratrol into the model membranes are shown as a change of surface pressure ($\Delta\pi$) over time after injection of resveratrol in Fig. 4.

As can be seen, after the resveratrol solution is injected into the subphase, the surface pressure increases to a maximum, then gently decreases and stabilizes over time. It is worth noting that the peaks in the above curves appear immediately after the injection of resveratrol into the subphase and may be related to the change in the molecular organization of the monolayers (i.e. the change in the orientation of the hydrophilic and hydrophobic groups of lipid molecules, as well as the reorientation of resveratrol molecules), which after some time reaches an equilibrium state. These results indicate that resveratrol molecules penetrate model membranes and become embedded between lipid molecules. It is also worth noting that the greatest changes in surface pressure are observed for the cholesterol-free membrane and $\Delta\pi$ decreases as the cholesterol concentration increases. This indicates that increasing content of cholesterol in the lipid membrane hinders the incorporation of resveratrol molecules contained in the subphase. These results are consistent with those obtained from the π-A isotherms and demonstrate that resveratrol has a higher affinity for more fluid monolayers, which decreases as the cholesterol content in the membrane increases.

Since lipid monolayers are a simplified model of the lipid membrane and are also not applicable in the study of transmembrane processes (e.g. in membrane permeability studies), in our research we used a more realistic membrane model, i.e. large unilamellar liposomes (LUVs) with the same composition as the investigated Langmuir monolayers. We have observed that the incubation of the obtained liposomes with resveratrol (in the range of the tested concentrations) does not change the hydrodynamic diameter of the liposomes or the dispersity index (please see Table S1 in the supplementary materials), which means that the incorporation of resveratrol into the liposome membranes does not change the size of the liposomes themselves or the thickness of their hydration shell, and also does not induce liposome aggregation.

In order to examine the affinity of resveratrol to the tested lipid membranes, we determined the values of the resveratrol partition coefficient between the lipid phase (LUV) and the aqueous PBS solution. The results obtained are shown in Fig. 5.

When the trend of changes in the partition coefficient value is analyzed depending on the cholesterol content in the sample, it can be seen that it is fully consistent with the results obtained previously presented for monolayers with the same lipid composition. Studies performed for liposomal suspensions also show that the affinity of resveratrol for the lipid membrane is closely related to its fluidity, which in this case is determined by the cholesterol content in the membrane. The results also indicate the protective nature of cholesterol, which, by increasing the order and packing of the lipids, protects the membrane against excessive penetration of substances from the external environment.

To verify the effect of resveratrol on the fluidity of the lipid bilayers, fluorescence anisotropy measurements were performed using DPH (1,6-diphenyl-1,3,5-hexatriene) as a fluorescent probe. Changes in the fluorescence anisotropy of DPH for mixed liposomes containing various amounts of cholesterol are presented in Fig. 6, while the results obtained for one-component POPC and SM liposomes are included in the supplementary materials (Fig. S2).

Comparing the values of fluorescence anisotropy obtained for liposomes dispersed in pure PBS solution (without resveratrol), it can be seen that the lowest anisotropy is observed for POPC/SM two-component liposomes and increases with increasing cholesterol content. Because DPH is a fluorescence probe located in the hydrophobic region of the bilayer [38–40], it can be concluded that POPC/SM liposomes are characterized by the greatest disorder of the acyl chains and thus the highest membrane fluidity among the investigated systems. The introduction of cholesterol into such a system increases the value of DPH

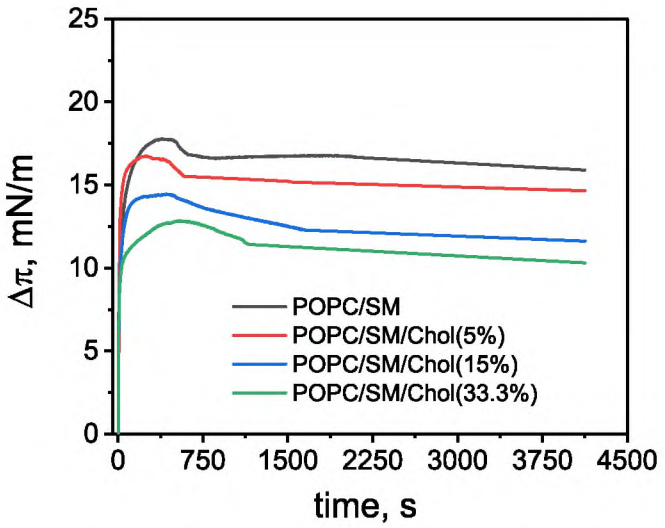

Fig. 4. Changes in surface pressure ($\Delta\pi$) over time due to resveratrol penetration into investigated monolayers at 30 mN/m (C_{Res} = 50 μM).

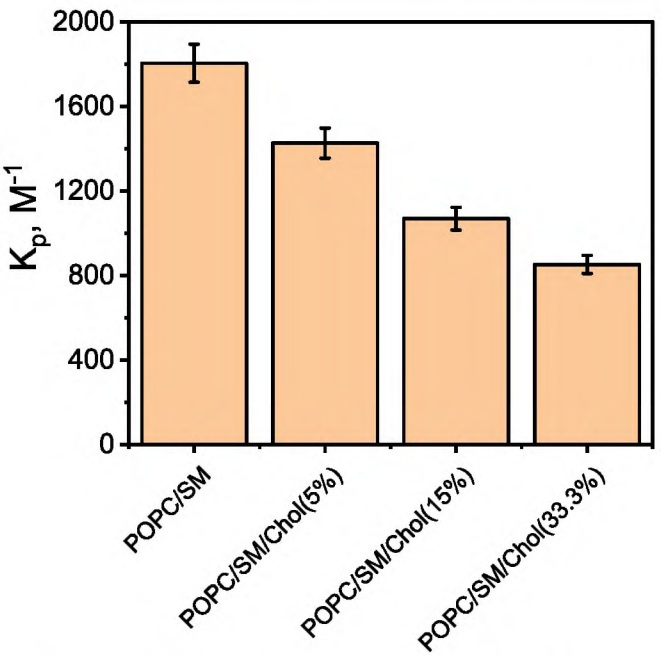

Fig. 5. Partition coefficients of resveratrol between the lipid and aqueous phases (K_p) for the tested suspensions of LUV liposomes depending on their composition at 20 °C.

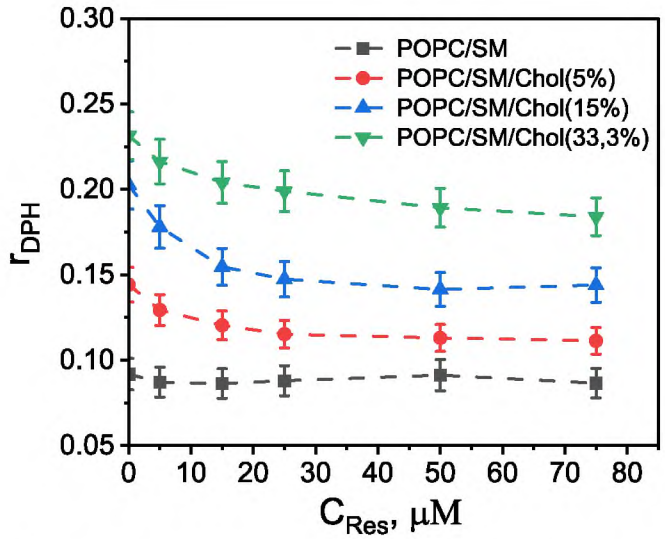

Fig. 6. Changes in steady-state fluorescence anisotropy of DPH (r_{DPH}) with the increase of resveratrol concentration for the investigated liposome membrane. Error bars represent the maximum values of standard deviations (SD) calculated from three independent measurements. The dashed lines that connect the points are not a fitted function, but are only intended to facilitate tracking the course of dependencies.

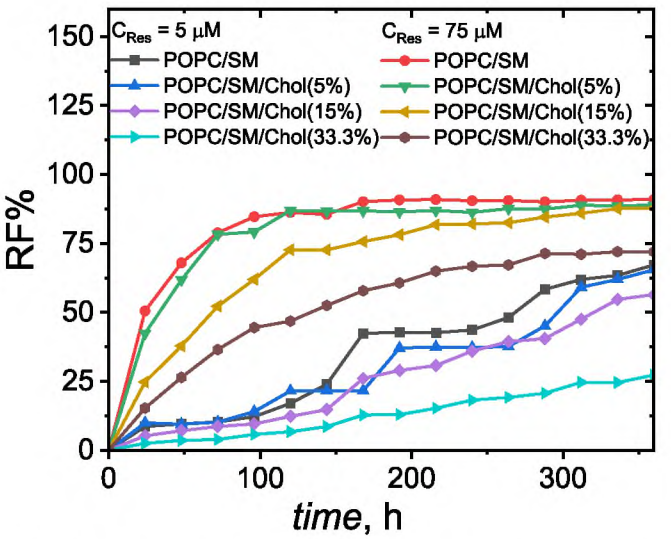

Fig. 7. The time-course of calcein release from the investigated liposomes at 20 °C. The solid lines that connect the points are not a fitted function, but are only intended to facilitate tracking the course of dependencies.

fluorescence anisotropy, which is related to the ordering and condensing effect that cholesterol molecules exert on the acyl chains of phospholipids [36,37]. This increase in the order and packing density of the molecules causes the stiffness of the bilayer to increase with an increase in the cholesterol content.

The addition of resveratrol to the PBS solution containing POPC/SM/Chol mixed liposomes causes a gradual decrease in the value of DPH fluorescence anisotropy, which stabilizes at higher resveratrol concentrations. This means that incorporation of resveratrol into the lipid membrane is accompanied by an increase in the disorder of the hydrophobic core of the membrane and thus an increase in the fluidity of the membrane. Changes in DPH anisotropy values, caused by the addition of resveratrol, practically are not observed for the POPC/SM system. The reason is that the lipid acyl chains in these liposomes are so disordered that the DPH molecule can move almost completely and is insensitive to the additional increase in membrane fluidity. The above conclusion regarding the fluidizing effect of resveratrol on the investigated lipid membranes is analogous to that drawn from the changes in the compressibility modulus observed for monolayers with the same lipid composition. It is worth noting, however, that the value of the compressibility modulus is determined by the global compressibility (fluidity) of the monolayer, which is influenced by both the hydrophilic and hydrophobic part of the monolayer, while the DPH fluorescence anisotropy indicates the degree of fluidity of the hydrophobic core of the membrane. Of course, the fluidity of the hydrophobic interior of the membrane can also be influenced by the packing of the hydrophilic groups of the lipids, which can to some extent indirectly be reflected in the fluorescence anisotropy values of the DPH probe.

The final stage of our research was to investigate the effect of resveratrol on the permeability of lipid membranes. For this purpose, liposome-encapsulated calcein release tests were performed. The results for resveratrol concentration of 5 μM and 75 μM were presented in Fig. 7.

Comparison of curves for systems with the same lipid composition and different resveratrol content indicates that an increase in resveratrol concentration results in faster release of calcein from the interior of the liposomes. This is due to the fluidizing effect of resveratrol on the lipid bilayer. This effect is probably a consequence of the weakening of the intermolecular interactions between lipids as a result of their separation by the incorporated resveratrol molecules, which makes the membrane more permeable to hydrophilic molecules. It is also worth noting that this effect is prevented by cholesterol, the presence of which in the membrane reduces the membrane's susceptibility to incorporation of resveratrol molecules, and additionally seals the lipid membrane, making it difficult for hydrophilic substances to pass through it.

5. Discussion and conclusions

Because of its potential health benefits, resveratrol has become the subject of numerous scientific studies. The health-promoting mechanism of action of resveratrol is not precisely known but it is suspected that one of its steps may be the effect of this compound on lipid membranes. Therefore, more and more studies are appearing on the interaction of resveratrol with lipid membranes using a variety of experimental techniques [41–52]. Although all the results of the studies prove that resveratrol is incorporated into the membrane, there is still an ongoing debate regarding its location, orientation and the effect it has on the physicochemical properties of lipid membranes. Some researchers indicate that resveratrol builds into the hydrophobic core of the lipid bilayer and orientates itself along the hydrophobic lipid chains thus exerting an ordering effect on the lipid chains, as cholesterol does. These authors postulate that reveratrol's ordering effect leads to the formation of highly ordered resveratrol-rich domains and phase separation within the membrane [41,43]. Other researchers, on the other hand, believe that resveratrol accumulates mainly in the region of hydrophilic groups [42,44,47,50,52] adopting a more tilted orientation [52], leading to an increase in fluidity of the lipid membrane [44,47,51,52].

In order to take a stand in the discussion of the effects of resveratrol on biological model membranes, we conducted studies using two complementary model systems, namely Langmuir monolayers and liposomes, which are very widely used model systems in the research on the properties of biomembranes [2,32,34,35,53–55]. Both monolayers and liposomes contained naturally occurring lipids in mammalian cell membranes, that is, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), sphingomyelin (SM) and cholesterol (Chol) with a constant ratio of POPC to SM and increasing cholesterol content (5, 15, 33.3 mol %). All tested systems were subjected to an increasing concentration of resveratrol.

Studies on monolayer systems clearly indicate that resveratrol present in the aqueous subphase is incorporated into all the tested lipid films, which is manifested in the shift of π-A isotherms toward larger

average surfaces per molecule, and this effect is greater the higher the concentration of resveratrol and the more the monolayer is fluid. In addition, among the POPC/SM/Chol ternary monolayers, the smallest shift of isotherms under the influence of resveratrol is observed for the monolayer containing the largest amount of cholesterol. This is also confirmed by the results of the study of resveratrol penetration into monolayers with different cholesterol content, which prove that resveratrol is most strongly incorporated into the POPC/SM monolayer, which does not contain cholesterol, and the weakest into the mixed POPC/SM/Chol film with the highest cholesterol content (33.3 mol%). This means that the increasing cholesterol content prevents the incorporation of resveratrol molecules into the lipid monolayer. The above observation is also confirmed by the values of the resveratrol partition coefficient between the lipid and aqueous phases determined for the tested liposomal formulations, which are the lower the higher the cholesterol content in the bilayer. This is the result of the ordering and condensing effect that cholesterol has on phospholipid membranes, which significantly hinders the incorporation of resveratrol molecules present in the aqueous phase into the membrane.

The results obtained also indicate that the incorporation of resveratrol is accompanied by an increase in membrane fluidity. This is manifested in both the decreasing values of the compressibility modulus for the monolayers and the decreasing values of the fluorescence anisotropy of the DPH probe observed for the tested liposome systems. This means that the incorporation of resveratrol into the membrane leads to loosening of the lipid packing and increases the disorder of the lipid acyl chains. The studies conducted also indicate that the incorporation of resveratrol into the membrane leads to an increase in its permeability, which is the result of an increase in the disorder of lipids in the membrane and thus an increase in its fluidity.

The results of our research showing the fluidizing effect of resveratrol on lipid membranes seem to contradict the results obtained by other authors who believe that resveratrol is localized in the membrane core and exerts an ordering effect on lipids. These authors, following synchrotron X-ray scattering and Forster energy transfer resonance (FRET) studies [41,43], showed that resveratrol induces a phase separation between the liquid-ordered phase (l_o) and the liquid-disordered phase (l_d). In addition, using measurements of DPH fluorescence quenching by TEMPO and tests of the resistance of lipid bilayers to detergent action (Triton X-100), they suggested that the ordering and condensation of the membrane increases with increasing resveratrol concentration, which significantly hinders the quenching of DPH fluorescence by TEMPO and increases the membrane resistance to triton X-100 [41]. It is worth noting, however, that in research on fluorescence quenching and resistance detergent assay, the authors did not take into account that the weaker fluorescence quenching, as well as the higher concentration of triton X-100, necessary for disintegration of the membrane, may be the result of the interactions of TEMPO and triton X-100 with resveratrol in the aqueous phase, which concentration increases and additionally depends on the examined system due to a different degree of membrane penetration depending on its composition. The ordering effect of resveratrol on lipid membranes, similar to that of cholesterol, is rather unlikely if the structures of these compounds are compared. Resveratrol, unlike cholesterol, has three hydrophilic groups, which are located on both sides of the molecule. It seems reasonable, therefore, that resveratrol will accumulate in the region of hydrophilic groups by adopting an orientation that allows the formation of hydrogen bonds between the -OH groups of resveratrol and the hydrophilic groups of lipids. As a result, the incorporation of resveratrol molecules should be accompanied by an increase in membrane surface area and fluidity. A decrease in membrane ordering with increasing resveratrol concentration has been found by several authors in studies using differential scanning calorimetry (DSC), in which a decrease in the phase transition temperature from the gel state to the liquid crystalline state (T_m) was observed [51,52]. This effect was also observed in studies of the fluorescence anisotropy of TMA-DPH embedded in the DPPC membrane, which is lower in the presence of resveratrol [47]. This is also confirmed by the results obtained by molecular dynamics methods, which show that resveratrol incorporates mainly in the hydrophilic group region, and its molecules prefer to be tilted, with the most favorable angle of 75 degrees along the normal membrane, which increases the surface area of the lipid bilayer, reduces its ordering and reduces its thickness [52]. Also, our results for lipid monolayers and bilayers clearly indicate that resveratrol increases membrane fluidity. This does not mean, however, that the studies indicating that resveratrol induces phase separation between the l_o and l_d states are wrong, but that the mechanism is opposite i.e. resveratrol does not participate in the formation of ordered domains but induces the formation of a disordered liquid phase that separates from the ordered domains containing cholesterol and/or saturated lipids. Taking into account the above results, it seems that the resveratrol-induced increase in membrane fluidity and decrease in its thickness should also be accompanied by an increase in its permeability to hydrophilic substances, which is confirmed by our results. However, it seems unlikely that resveratrol reduces membrane permeability to water molecules, as observed by J. Ceja-Vega et al. [49]. Perhaps the decrease in membrane permeability observed by them is the result of the design of their measuring system, in which lipid molecules adsorbed on drops of aqueous solutions as well as resveratrol molecules additionally interact with the squalene in which they are placed, which, however, requires further research.

In conclusion, our studies indicate that resveratrol has the ability to build into lipid membranes, leading to a change in their physicochemical properties. These changes include, above all, an increase in membrane fluidity, which in turn may lead to disturbances in the activity of transmembrane proteins or their translocation. Similarly, the incorporation of resveratrol into lipid rafts can disrupt their functioning. Changing the activity of lipid rafts and membrane proteins by increasing the fluidity of the lipid matrix may be one of the possible mechanisms of action of resveratrol. In addition, our research may contribute to the development of the optimal composition of liposomes, which can be effective carriers of resveratrol.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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