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β-sitosterol and gentisic acid loaded 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine liposomal particles

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Abstract

The aim of the present study was the examination of the impact of β-sitosterol and gentisic acid on the characteristics of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) liposomal particles: (a) bilayer permeability (fluorescence spectroscopy), (b) particle size, polydispersity index (PDI) and zeta potential (photon correlation spectroscopy) and (c) thermal properties (differential scanning calorimetry). β-sitosterol induced the increase of liposomal bilayer rigidity, due to rearranging of the phospholipid chains, while gentisic acid enhanced the membrane fluidity, due to the reduced orderliness and the increase of phospholipid dynamics. The inclusion of β-sitosterol in liposomes caused a significant increase in particle diameter and PDI, while the encapsulation of gentisic acid did not have influence on particle size distribution. Apart from that, the presence of β-sitosterol resulted in the significant zeta potential increase, and thus a better stability of liposomal spheres (in the absence and in the presence of gentisic acid). β-sitosterol decreased main transition temperature (T_m) and phase transition enthalpy (ΔH), and caused the disappearance of the pre-transition peak as well, whereas the presence of gentisic acid produced a slight decrease in T_m and increase of ΔH. Therefore, gentisic acid had more favourable, stabilizing interactions with phospholipids than β-sitosterol. Thus, it can be concluded that β-sitosterol is located in the bilayer interior between phospholipids acyl chains, and gentisic acid is incorporated near the outer leaflet of the phospholipid membrane, next to the polar head groups. β-sitosterol and gentisic acid loaded DPPC liposomal particles have a potential to be used in food and pharmaceutical products, due to the important individual and possible synergistic beneficial health properties of β-sitosterol and gentisic acid.

Keywords: β-sitosterol, gentisic acid, liposomes, permeability.

1. INTRODUCTION

Liposomes represent spherical micrometric or nanometric particles comprising a central aqueous compartment, encircled by one or more bilayers which are composed mainly of phospholipids and sterols. Biocompatible and biodegradable liposomal particles are suitable for delivery of various bioactive components, including vitamins, polyphenols, carotenoids, peptides, enzymes, essential oils (Hammoud, Gharib, Fourmentin, Elaissari, & Greige-Gerges 2019; Jovanović et al. 2018; Reidel, Camussone, Archilla, Calvinho, & Veaute 2019). During the encapsu-

lation of active principles in liposomal vesicles, lipophilic compounds can be located within the lipid bilayer, hydrophilic substances in the aqueous internal cavity, whereas amphiphilic components are found at the water-lipids interface (Hammoud et al. 2019). 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), the naturally occurring zwitterionic lipid (molecular weight of 734.1 g/mol and molecular formula $C_{40}H_{80}NO_8P$, Figure 1a), is frequently used for the preparation of liposomes, as model systems of cell membranes or as particles for the delivery of active substances (Neunert et al. 2018; Puskás & Csem-

pesz 2007; Ricci et al. 2016). Apart from that, with the aim to fill the gaps into liposomal bilayers, as well as to reduce leakage of active principles, sterols are usually added into the mixture for the preparation of liposomes. Therefore, this step can influence the fluidity and stability of the liposomal membrane (Chen, Han, Cai, & Tang 2010). Phytosterols, β-sitosterol included (Figure 1a), are similar in structure and function to animal sterols and therefore can be used in liposomes formulations. Moreover, phytosterols demonstrate numerous health benefits, including lowering plasma cholesterol and triacylglyceride levels and reducing the risk of cardiovascular diseases (Quilez, Garcia-Lorda, & Salas-Salvado 2003). According to the literature, gentisic acid (as a phenolic acid, Figure 1c) has show chemopreventive effect against breast and brain cancers (Altinoz, Elmaci, Cengiz, Emekli-Alturfan, & Ozpinar 2018), while plant extracts rich in this active substance have exerted antiproliferative and apoptotic effect on glioma cell cultures (Sitarek et al. 2017). However, phenolic acids are quite sensitive to light, high temperature, oxygen and the presence of different pH, enzymes and nutrients in the gastrointestinal tract (Munin & Edwards-Lévy 2011; Pasukamonset, Kwon, & Adisakwattana 2016). Thus, in order to enhance stability, bioavailability and efficacy of active compounds, as well as to achieve their prolonged release, numerous carriers (such as liposomes) are developed and used (Djorđević et al. 2015; Eloy et al. 2016; Jovanović et al. 2019; Munin & Edwards-Lévy 2011).
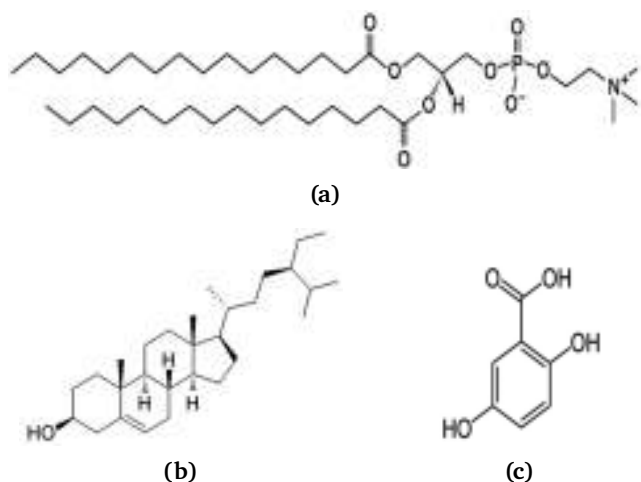
Figure 1. Structure of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine-DPPC (A), β-sitosterol (B) and gentisic acid (C)

The present study investigates the influence of β-sitosterol and gentisic acid on the characteristics of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) liposomes, including bilayer permeability (fluorescence spectroscopy), particle size, polydispersity index (PDI) and zeta potential (photon correlation spectroscopy) and thermal properties (differential scanning calorimetry, DSC).

2. MATERIAL AND METHODS

2.1. Chemicals

Lipid DPPC was purchased from Avanti Polar Lipids Inc. (USA), while β-sitosterol, chloroform, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 1,6 diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) were purchased from Sigma-Aldrich (USA).

2.2. Preparation of DPPC liposomes

DPPC liposomes were prepared by the following method: the required amounts of chloroform solutions of DPPC lipid and β-sitosterol (0 or 10 mol %) and methanol solution of gentisic acid ($n_{GA}/n_{lip} = 10^{-1}$) were evaporated (Rotavapor R-210, Büchi, Switzerland, pressure 17 mbar, 45°C, 3 h) and the thin lipid film was obtained (Eloy et al. 2016). DPPC-β-sitosterol liposomes (without gentisic acid) were prepared as well. Further, in order to form multilamellar vesicles (MLVs), the thin film was hydrated using HEPES buffer and the final concentration of lipids in liposomal suspension was 2 mg/mL. Small unilamellar vesicles (SUVs) were prepared from MLVs using ultrasound waves (titanium probe, with 10 s *on-off* cycles for 30 min and amplitude of 40%).

2.3. Fluorescence polarization and anisotropy measurements

Fluorescence polarization and anisotropy measurements were performed in a fluorescence spectrophotometer (Cary Eclipse, Varian, Austria). Liposomal suspension of SUVs (DPPC and DPPC-β-sitosterol liposomes without gentisic acid) was diluted by HEPES and the lipid concentration in the samples was 0.1 mg/mL. With the aim to analyze the fluidity of DPPC and DPPC-β-sitosterol liposomes, DPH fluorophore was mixed with SUVs, and polarization (p) and anisotropy (r) were measured within temperature range from 20 to 60°C. Apart from that, in order to determine the influence of encapsulated gentisic acid on liposomal membrane fluidity, the mixture of diluted liposomal suspension and TMA-DPH fluorophore was titrated by the addition of gentisic acid aliquots (n_{GA}/n_{lip} varied from 0 to 1) or water as a control, at 25°C. DPH and TMA-DPH fluorescence polarization values were measured at the excitation wavelength of 358 nm and the emission wavelength of 410 nm. Each sample was measured four times.

2.4. Measurements of liposome particle size, PDI and zeta potential

Photon correlation spectroscopy was employed for determination of particle size, PDI and zeta potential of MLVs and SUVs, at 25°C, using Zetasizer Nano Series and Zetasizer Software (Malvern, UK). All measurements were done in triplicate.

2.5. Analysis of liposomes thermal properties

The thermal properties (transition temperature and enthalpy) of the DPPC and DPPC-β-sitosterol liposomal particles (in the absence and in the presence of gentisic acid, $n_{GA}/n_{lip} = 10^{-1}$) were analyzed by using NANO DSC III instrument (Calorimetry Science, USA). The liposomal samples (MLVs) and HEPES buffer (control) were degassed under vacuum. Subsequently, the liposomal suspension (1 mL, 1 mg/mL) and HEPES were placed in the calorimetry cells. The gel-to-liquid phase transition was monitored within the temperature range from 10 to 70°C.

3. RESULTS AND DISCUSSION

3.1. Polarization and anisotropy

With the aim to examine the influence of β-sitosterol and gentisic acid on the permeability of DPPC liposomal membrane, fluorescence measurements were performed by using two kinds of fluorophores, DPH (for influence of β-sitosterol) and TMA-DPH (for influence of gentisic acid). These mentioned fluorescence probes are easily inserted into the hydrophobic region of the lipid bilayer, with the fact that DPH is positioned at the interior of the membrane, whereas TMA-DPH is located around the membrane surface (Balanč et al. 2015). Therefore, DPH and TMA-DPH fluorescence is influenced by the polar environment of the liposomal bilayer. The obtained results of fluorescence polarization and anisotropy measurements of DPH in DPPC and DPPC-β-sitosterol liposomal suspension (without gentisic acid) are shown in Figure 2 (A and B, respectively).

As can be seen from Figure 2, polarization and anisotropy values (p and r, respectively) have decreased with the increase of the temperature (20–60°C).

In the case of pure DPPC liposomes, p values changed from 0.455 to 0.099, whereas r values were 0.360 at 20°C and 0.069 at 60°C. The obtained results had been expected since high temperatures enhanced the movements of phospholipids and thus increased the permeability of the liposomal membrane. Moreover, a significant drop in p and r values at 45°C could be noticed, which was in accordance with the literature data and with the results obtained in DSC analysis as well (Paragraph 3.3); for each
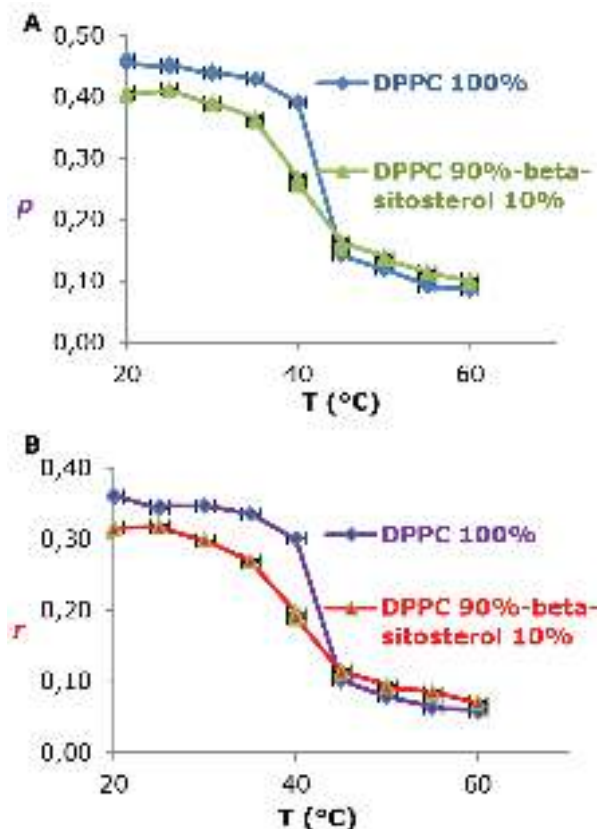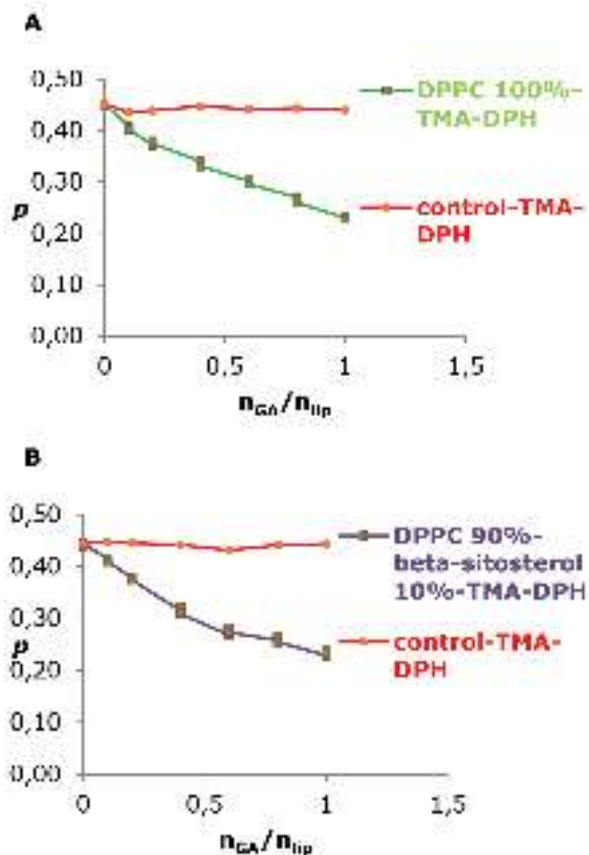

Figure 2. Fluorescence polarization-p (A) and anisotropy-r (B) of 1,6 diphenyl-1,3,5-hexatriene (DPH) added to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and DPPC-β-sitosterol liposomes, at different temperatures (20–60°C)

phospholipid, p and r values are high in the gel phase and decrease significantly above the phase transition temperature (McMullen & McElhaney 1995; Wu et al. 2012). The same trend was noticed in the sample with DPPC-β-sitosterol system, except that p and r values were lower in comparison to pure DPPC liposomes, but only up to the temperature of 40°C. After that, values were higher in the case of DPPC-β-sitosterol liposomes. This could be explained by the restricted mobility of DPH fluorophore and rigid/ordered bilayer membrane in the system containing β-sitosterol, even at higher temperatures. Also, it was noticeable that the addition of β-sitosterol (had) caused the slight drop in p and r values, but already above 35°C, which was consistent with the literature and with the data obtained in DSC analysis (Paragraph 3.3); sterols rearranged the phospholipids chains making the membrane less fluid and at the same time decreasing the phase transition temperature (Ricci et al. 2016; Wu et al. 2012).

The results of fluorescence polarization measurements of TMA-DPH in DPPC and DPPC-β-sitosterol liposomal suspension titrated by the addition of gentisic acid are presented in Figure 3 (A and B, respectively).

According to the graphs in Figure 3, with the increase of gentisic acid content (n_{GA}/n_{lip} 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1), p values gradually decreased, from 0.435 to 0.233 in DPPC system and from 0.432 to 0.228 in DPPC-β-sitosterol liposomes. Therefore, the addition of gentisic acid caused the enhancement of the permeability of the liposomal bilayer. These results indicated that gentisic acid decreased the ordering and increased the dynamics of phospholipid alkyl chains in the TMA-DPH environment, and thus, the bilayer became less rigid when more gentisic acid was incorporated into liposomes.

Figure 3. Fluorescence polarization (p) of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) added to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and DPPC-β-sitosterol liposomes and titrated with gentisic acid (GA)

Poklar Ulrih et al. (2010) showed that higher concentrations of polyphenols caused the increase in liposomal membrane fluidity as well. The introduction of $-$OH group at the *o*-position in benzoic acid (as in the case of gentisic acid, Figure 1) resulted in the changes of cell membrane permeability, as well as in the increase of osmotic fragility (Mineo et al. 2013). On the other hand, since there is no significant difference between p values of DPPC and DPPC-β-sitosterol liposomes containing gentisic acid, it can be concluded that the presence of β-sitosterol does not induce alteration or disturbance during incorporation of gentisic acid, probably because of low β-sitosterol content (10 mol %).

3.2. Particle size, PDI and zeta potential

The particle size, PDI and zeta potential of the liposomal vesicles were measured using photon correlation spectroscopy at room temperature. Table 1 represents the mean particle size, PDI and zeta potential of DPPC and DPPC-β-sitosterol liposomes, in the absence and in the presence of gentisic acid.

As can be seen from Table 1, the inclusion of β-sitosterol in liposomal structure causes significant changes both in MLVs size and SUVs size (the increase of liposomal vesicle diameter). The reason could lie in the interaction of sterols with phospholipids chains, formation of the inter-lipid space and consequently liposomal membrane expansion. As mentioned earlier, the presence of sterols in the bilayer produced a rigid liposomal membrane, and thus caused the decrease in particle deformability, which also led to the greater vesicle size. However, all the obtained MLVs were quite large, which could be attributed to the thin film method used for liposomal preparation; the application of ultrasound waves induced significant particle size reduction, approximately 10 times in the case of DPPC liposomes and 17–20 times in the case of DPPC-β-sitosterol system. Also, it was noticeable that the presence of gentisic acid did not cause significant changes in MLVs and SUVs size. PDI, as a measure of the vesicle size distribution, can range from 0, related to monodisperse system, to 1, related to heterodisperse system. β-sitosterol induced an increment of PDI values (the increase of particle heterogeneity), particularly in the case of SUVs, whereas the addition of gentisic acid did not have that effect (Table 1).

Interestingly, the ultrasound waves induced the enhancement of heterogeneity (higher PDI values) as well, probably because of the viscosity of liposomal suspension. Namely, the presence of a large amount of lipids and sterols contributed to the attenuation of the ultrasound waves and the active part was restricted to a zone located in the vicinity of the ultrasound probe (Jovanović et al. 2017; Wang & Weller 2006). Thus only vesicles around probe could have been significantly reduced, while the influence of ultrasound waves on the rest of the spheres was quite minor. Regarding the data obtained by measuring the zeta potential, DPPC liposomes (in the absence and in the presence of gentisic acid)showed a significantly lower value of zeta potential (Table 1). On the other hand, the inclusion of β-sitosterol caused a significant enhancement of zeta potential, and consequently a better stability of liposomal particles, due to the increase of the space

Table 1. Particles size, polydispersity index (PDI) and zeta potential of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and DPPC-β-sitosterol liposomes (multilamellar vesicles-MLVs and small unilamellar vesicles-SUVs), in the absence and in the presence of gentisic acid (GA), at 25°C

	Sample	particles size (nm)	PDI	zeta potential (mV)
MLVs[1]	DPPC	1146.0 ± 33.0	0.138 ± 0.033	2.95 ± 0.43
	DPPC-β-sito	2444.7 ± 86.5	0.251 ± 0.026	15.7 ± 0.1
	DPPC +GA	1119.7 ± 48.0	0.178 ± 0.062	4.06 ± 0.62
	DPPC-β-sito +GA	2399.1 ± 63.4	0.254 ± 0.031	18.3 ± 0.5
SUVs	DPPC	107.2 ± 0.2	0.290 ± 0.020	1.58 ± 0.39
	DPPC-β-sito	127.6 ± 2.1	1.0 ± 0.0	5.56 ± 0.40
	DPPC +GA	113.8 ± 3.1	0.335 ± 0.081	1.74 ± 0.22
	DPPC-β-sito +GA	142.7 ± 1.9	0.495 ± 0.030	6.12 ± 0.18

between lipid head group and hydrophobic stabilization of liposomes (Bhattacharya & Haldar 2000). Apart from that, the addition of β-sitosterol induced changes in the phospholipid order and provided the hydrogen-bonding interactions (Bhattacharya & Haldar 2000), which led to the zeta potential change; values for DPPC liposomes were 2.95 ± 0.43 (in the absence of gentisic acid) and 4.06±0.62 (in the presence of gentisic acid), whereas liposomes containing sterol possessed higher absolute value, −15.7 ± 0.1 mV for DPPC-β-sitosterol and −18.3 ± 0.5 mV for DPPC-β-sitosterol with gentisic acid. According to the data presented in Table 1, the reduction of particle size, from MLVs to SUVs, led to the significant decrease in surface charge of both DPPC and DPPC-β-sitosterol, particularly in the case of liposomes containing sterol. Thus it could be concluded that SUVs represented unstable liposomes. Nevertheless, SUVs with incorporated β-sitosterol were more stable compared to SUVs without sterol. All mentioned results point to an increase in the stability of liposomes with incorporated β-sitosterol, since the more stable colloid dispersion is defined with a higher absolute value of zeta potential (Honary & Zahir 2013). Furthermore, only in MLVs, gentisic acid caused a low, but still significant increase in zeta potential. Since encapsulated active principles, such is gentisic acid, induce changes in membrane permeability, they can change the order in phospholipid chains, as well as the value of total charge (Bhattacharya & Haldar 2000; Mineo et al. 2013; Ulrih et al. 2010).

3.3. Thermotropic properties of liposomal particles

Thermotropic characteristics (temperature of the phase transition-T_m and the changes in enthalpy-ΔH) of the DPPC and DPPC-β-sitosterol liposomes, in both the absence and the presence of gentisic acid, were monitored by using DSC. The thermograms are presented in Figure 4.
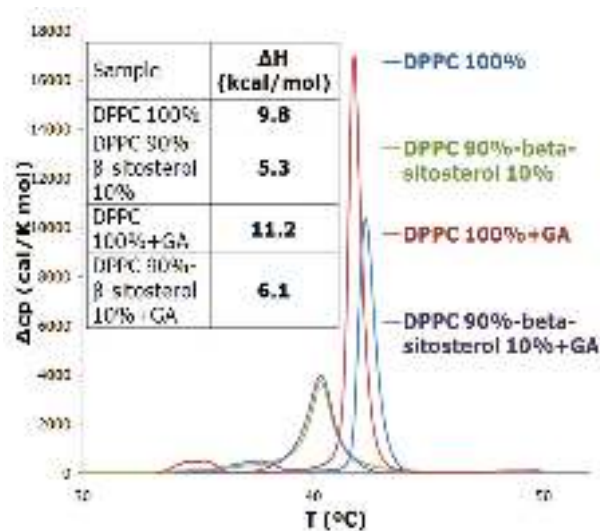
Figure 4. DSC thermograms of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and DPPC-β-sitosterol liposomes in the absence and in the presence of gentisic acid (GA); ΔH, phase transition enthalpy; DSC, differential scanning calorimetry

As can be seen from the thermogram of pure DPPC liposomes (without β-sitosterol and gentisic acid, Figure 4), a low and wide pre-transition peak appeared at 37.7°C (T_{pre}), while the main and sharp transition peak was at 42.3°C (T_m). The obtained values of pre-transition and main transition temperatures of pure DPPC liposomes are in good agreement with literature data (Balanč et al. 2015). According to the literature, the pre-transition represents conversion of a lamellar gel phase to a rippled gel phase, relating to the polar phospholipids area, and the main phase transition, as a conversion of a rippled gel phase to a liquid crystal phase, represents the changes in the acid chains of phospholipids and interactions with encapsulated compounds (Ricci et al. 2016; Wu et al. 2012). However, on the thermogram of 100% DPPC liposomal particles with incorporated gentisic acid ($n_{GA}/n_{lip} = 10^{-1}$), T_{pre} is seen at 35.3°C, whereas T_m ap-

pears at 41.7°C. Although, according to the literature, the encapsulation of active compounds resulted in the disappearance of the pre-transition peak (Balanč et al. 2015), in the case of DPPC liposomes containing gentisic acid the mentioned peak existed. It could be explained by the presence of a lower content of active substance (lipid/gentisic acid molar ratio was only 10^{-1}). Nevertheless, the incorporation of gentisic acid into DPPC liposomes caused a slight decrease in T_m and increase in ΔH (from 9.8 kcal/mol for pure DPPC to 11.2 kcal/mol for DPPC with gentisic acid).

The presented results are in agreement with the literature data given that Wu et al. (2012) have shown that high ΔH indicated the presence of stabilizing interactions between encapsulated substances and lipids. Furthermore, it can be noticed that the presence of β-sitosterol in the DPPC liposomal bilayer caused the disappearance of the pre-transition peak (both in the absence and in the presence of gentisic acid, Figure 4). Several authors have reported that in the liposomal particles containing sterols (at concentrations above 5 mol %) the pre-transition temperature peak does not exist (Ricci et al. 2016; Wu et al. 2012). The reason can be in the presence of a rippled gel phase at room temperature, due to the inclusion of β-sitosterol into the bilayer structure. As can be seen from Figure 4, the main phase transition temperature and enthalpy of liposomes containing 10 mol % of β-sitosterol were lower than the same parameters of liposomes without sterol. Namely, the addition of β-sitosterol induced a decrease in T_m (40.3°C, both in the absence and in the presence of gentisic acid), whereas ΔH values were 5.3 and 6.1 kcal/mol in the absence and in the presence of gentisic acid, respectively; β-sitosterol caused a significant decrease in enthalpy compared to pure DPPC liposomes, which is in agreement with previous studies (Jovanović et al. 2018; Wu et al. 2012). Comparing the incorporation of β-sitosterol and gentisic acid into liposomal particles, it could be concluded (*via* the changes in enthalpy) that gentisic acid had more favourable, stabilizing interactions with phospholipids than β-sitosterol. Moreover, according to the presented results, it can be concluded that gentisic acid is located near the outer leaflet of the liposomal bilayer, next to the polar head groups, whereas β-sitosterol occupies the membrane interior between phospholipids acyl chains.

4. CONCLUSION

In the present research, β-sitosterol and gentisic acid were used for the preparation of DPPC multilamellar and unilamellar particles, and their influence on liposomal bilayer permeability, particle size, PDI, zeta potential and thermotropic properties was investigated. Bilayer perme-

ability measurements indicated that β-sitosterol (incorporated in the interior of the membrane) rearranged the phospholipids chains making the membrane less fluid, whereas gentisic acid (occupied the membrane outer leaflet) enhanced the permeability of liposomal bilayer. The incorporation of β-sitosterol into liposomal structure resulted in the significant increase of liposomal vesicle diameter (both MLVs and SUVs), while the presence of gentisic acid did not cause significant changes in liposomal particle size. However, β-sitosterol caused the increase of particles heterogeneity, particularly in the case of SUVs; the encapsulation of gentisic acid did not have influence on PDI. Furthermore, the inclusion of β-sitosterol caused significant zeta potential increase, and thus a better stability of liposomal particles (in the absence and in the presence of gentisic acid). β-sitosterol showed the following influence of thermal characteristics of DPPC liposomal particles: the decrease of T_m and ΔH, as well as the disappearance of the pre-transition peak. On the other hand, gentisic acid exhibited the following impact on the thermotropic properties of DPPC liposomes: a slight decrease in main transition temperature and increase of phase transition enthalpy. Therefore, gentisic acid shows more favourable and stabilizing interactions with lipids than β-sitosterol. β-sitosterol and gentisic acid loaded DPPC liposomal particles, evaluated in the present study, have a potential to be used in food and pharmaceutical products, due to the important individual and possible synergistic beneficial health propertiesof β-sitosterol and gentisic acid.

CONFLICT OF INTEREST

The authors declare that they have no financial and commercial conflicts of interest.

REFERENCES

Altinoz, M. A., Elmaci, I., Cengiz, S., Emekli-Alturfan, E., & Ozpinar, A. (2018). From epidemiology to treatment: Aspirin's prevention of brain and breast-cancer and cardioprotection may associate with its metabolite gentisic acid. *Chemico-biological interactions, 291,* 29–39. doi: https://doi.org/10.1016/j.cbi.2018.05.016

Balanč, B. D., Ota, A., Djordjević, V. B., Šentjurc, M., Nedović, V. A., Bugarski, B. M., & Ulrih, N. P. (2015). Resveratrol-loaded liposomes: Interaction of resveratrol with phospholipids. *European journal of lipid science and technology*, *117*(10), 1615–1626. doi: https://doi.org/10.1002/ejlt.201400481

Bhattacharya, S., & Haldar, S. (2000). Interactions between cholesterol and lipids in bilayer membranes. role of lipid headgroup and hydrocarbon chain–backbone linkage. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1467*(1), 39–53. doi: https://doi.org/10.1016/S0005-2736(00)00196-6

Chen, C., Han, D., Cai, C., & Tang, X. (2010). An overview of liposome lyophilization and its future potential. *Journal of controlled release*, *142*(3), 299–311. doi: https://doi.org/10.1016/j.jconrel.2009.10.024

Djorđević, V., Balanč, B., Belščak-Cvitanović, A., Lević, S., Trifković, K., Kalušević, A., ... Nedović, V. (2015). Trends in encapsulation technologies for delivery of food bioactive compounds. *Food Engineering Reviews*, *7*(4), 452–490. doi: https://doi.org/10.1007/s12393-014-9106-7

Eloy, J. O., Petrilli, R., Topan, J. F., Antonio, H. M. R., Barcellos, J. P. A., Chesca, D. L., ... Marchetti, J. M. (2016). Co-loaded paclitaxel/rapamycin liposomes: development, characterization and in vitro and in vivo evaluation for breast cancer therapy. *Colloids and Surfaces B: Biointerfaces*, *141*, 74–82. doi: https://doi.org/10.1016/j.colsurfb.2016.01.032

Hammoud, Z., Gharib, R., Fourmentin, S., Elaissari, A., & Greige-Gerges, H. (2019). New findings on the incorporation of essential oil components into liposomes composed of lipoid s100 and cholesterol. *International journal of pharmaceutics*, *561*, 161–170. doi: https://doi.org/10.1016/j.ijpharm.2019.02.022

Honary, S., & Zahir, F. (2013). Effect of zeta potential on the properties of nano-drug delivery systems-a review (part 2). *Tropical Journal of Pharmaceutical Research*, *12*(2), 265–273. doi: http://doi.org/10.4314/tjpr.v12i2.20

Jovanović, A. A., Balanč, B. D., Djordjević, V. B., Ota, A., Skrt, M., Šavikin, K. P., ... Ulrih, N. P. (2019). Effect of gentisic acid on the structural-functional properties of liposomes incorporating *β*-sitosterol. *Colloids and Surfaces B: Biointerfaces*, *183*, 110422. doi: https://doi.org/10.1016/j.colsurfb.2019.110422

Jovanović, A. A., Balanč, B. D., Ota, A., Ahlin Grabnar, P., Djordjević, V. B., Šavikin, K. P., ... Poklar Ulrih, N. (2018). Comparative effects of cholesterol and *β*-sitosterol on the liposome membrane characteristics. *European Journal of Lipid Science and Technology*, *120*(9), 1–11. doi: https://doi.org/10.1002/ejlt.201800039

Jovanović, A. A., Djorđević, V. B., Zdunić, G. M., Pljevljakušić, D. S., Šavikin, K. P., Gođevac, D. M., & Bugarski, B. M. (2017). Optimization of the extraction process of polyphenols from *Thymus serpyllum* l. herb using maceration, heat-and ultrasound-assisted techniques. *Separation and Purification Technology*, *179*, 369–380. doi: https://doi.org/10.1016/j.seppur.2017.01.055

McMullen, T. P., & McElhaney, R. N. (1995). New aspects of the interaction of cholesterol with dipalmitoylphosphatidylcholine bilayers as revealed by high-sensitivity differential scanning calorimetry. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1234*(1), 90–98. doi: https://doi.org/10.1016/0005-2736(94)00266-r

Mineo, H., Ogita, A., Kanayama, N., Kawagishi, M., Sato, E., Yamamoto, N., ... Izawa, M.-a. (2013). Effect of the chemical specificity of benzoic acid and its analogs on osmotic fragility in erythrocytes of sprague-dawley rats in vitro. *European journal of pharmacology*, *702*(1-3), 142–148. doi: https://doi.org/10.1016/j.ejphar.2013.01.029

Munin, A., & Edwards-Lévy, F. (2011). Encapsulation of natural polyphenolic compounds; a review. *Pharmaceutics*, *3*(4), 793–829. doi: https://doi.org/10.3390/pharmaceutics3040793

Neunert, G., Tomaszewska-Gras, J., Siejak, P., Pietralik, Z., Kozak, M., & Polewski, K. (2018). Disruptive effect of tocopherol oxalate on dppc liposome structure: Dsc, saxs, and fluorescence anisotropy studies. *Chemistry and physics of lipids*, *216*, 104–113. doi: https://doi.org/10.1016/j.chemphyslip.2018.10.001

Pasukamonset, P., Kwon, O., & Adisakwattana, S. (2016). Alginate-based encapsulation of polyphenols from clitoria ternatea petal flower extract enhances stability and biological activity under simulated gastrointestinal conditions. *Food hydrocolloids*, *61*, 772–779. doi: https://doi.org/10.1016/j.foodhyd.2016.06.039

Puskás, I., & Csempesz, F. (2007). Influence of cyclodextrins on the physical stability of dppc-liposomes. *Colloids and surfaces B: biointerfaces*, *58*(2), 218–224. doi: https://doi.org/10.1016/j.colsurfb.2007.03.011

Quilez, J., Garcia-Lorda, P., & Salas-Salvado, J. (2003). Potential uses and benefits of phytosterols in diet: present situation and future directions. *Clinical Nutrition*, *22*(4), 343–351. doi: https://doi.org/10.1016/S0261-5614(03)00060-8

Reidel, I. G., Camussone, C., Archilla, G. A. S., Calvinho, L. F., & Veaute, C. (2019). Liposomal and cpg-odn formulation elicits strong humoral immune responses to recombinant staphylococcus aureus antigens in heifer calves. *Veterinary immunology and immunopathology*, *212*, 1–8. doi: https://doi.org/10.1016/j.vetimm.2019.04.011

Ricci, M., Oliva, R., Del Vecchio, P., Paolantoni, M., Morresi, A., & Sassi, P. (2016). Dmso-induced perturbation of thermotropic properties of cholesterol-containing dppc liposomes. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1858*(12), 3024–3031. doi: https://doi.org/10.1016/j.bbamem.2016.09.012

Sitarek, P., Skała, E., Toma, M., Wielanek, M., Szemraj, J., Skorski, T., ... others (2017). Transformed root extract of leonurus sibiricus induces apoptosis through intrinsic and extrinsic pathways in various grades of human glioma cells. *Pathology & Oncology Research*, *23*(3), 679–687. doi: https://doi.org/10.1007/s12253-016-0170-6

Ulrih, N. P., Ota, A., Šentjurc, M., Kure, S., & Abram, V. (2010). Flavonoids and cell membrane fluidity. *Food chemistry*, *121*(1), 78–84. doi: https://doi.org/10.1016/j.foodchem.2009.12.006

Wang, L., & Weller, C. L. (2006). Recent advances in extraction of nutraceuticals from plants. *Trends in Food Science & Technology, 17*(6), 300–312. doi: http://doi.org/10.1016/j.tifs.2005.12.004

Wu, R.-g., Dai, J.-d., Wu, F.-g., Zhang, X.-h., Li, W.-f., & Wang, Y.-r. (2012). Competitive molecular interaction among paeonol-loaded liposomes: differential scanning calorimetry and synchrotron x-ray diffraction studies. *International journal of pharmaceutics, 438*(1-2), 91–97. doi: http://doi.org/10.1016/j.ijpharm.2012.08.052