

The influence of membrane composition on the release of polyphenols from liposomes

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Abstract

Polyphenols are compounds which are widely studied due to their antioxidative and potential therapeutic properties. Systems for the controlled release of drugs offer a number of benefits comparing with traditional forms of medicines and because of that these systems are widely researched. The objective of this paper is to investigate the possibility of using liposomes as carriers of polyphenols and influence of the membrane composition on the release rate of encapsulated polyphenols. Experiments show how the membrane modification affects the mass transfer comparing to a conventional liposomes. Liposomes were modified with surfactants Tween 20 and Tween 60, and thyme tea extract was used as a source of polyphenols. The diffusion of polyphenols from thyme extract, dispersion of conventional liposomes and liposomes modified with Tween 20 and Tween 60 were studied using Franz diffusion cell. From the experimental data diffusion coefficients were determined for each of the systems, as well as the corresponding diffusion resistances. From the obtained results it can be concluded that the encapsulation of polyphenols in liposomes significantly slows diffusion, and with membrane modification can be achieved further slowing. The diffusion resistance of the liposome membrane modified with Tween 20 and Tween 60 is about 5 times higher compared to the diffusion of unencapsulated polyphenols from the thyme extract.

Keywords: diffusion coefficient, diffusion resistance, liposomes, membrane modification, polysorbate, tea polyphenols.

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In recent years researchers have paid particular attention to the biologically active ingredients, especially polyphenols, due to their positive effects on human health [1]. Polyphenols are secondary plant metabolites that are involved in a wide range of specialized physiological functions [2,3] and they are found to exhibit highly potent anti-oxidant activity [4]. Bioactive compounds of herbal plants such as antioxidants have shown to exhibit multifunctional and remedial properties that include anti-radical, anti-carcinogenic, anti-inflammatory effects, as well as the reduction of oxidative stress and cardio-protection [5–7]. Therefore, there is a considerable interest in new natural antioxidants, such as polyphenols, to replace the synthetic ones and thyme (*Thymus vulgaris* L.) leaves appeared to be a promising source [8]. Thyme is a medicinal herb belonging to the Lamiaceae family, cultivated worldwide for culinary, cosmetic and medical purposes. This species is known for its beneficial functions such as

antispasmodic, expectorant, antiseptic, antimicrobial and as mentioned, antioxidant [9,10]. The main phenolic compounds in thyme are: glycuronids of apigenin, luteolin, eriodictiol, luteolin glycosides, rosmarinic acid and quercitine [11–13].

Polyphenols are mainly isolated from plants using water extraction procedure [14,15] and since being natural antioxidants, they should be protected from the surrounding medium [16]. This can be done by encapsulation into the adequate system which would promote better product stability due to the isolation of active compounds from the detrimental effects of oxygen, moisture or incompatible compounds [5]. Encapsulation is used not only to protect active components, but also to trap and release them under controlled conditions [16]. Additionally, controlled delivery could enhance bioavailability of an active compound by customizing the release mechanism [5]. As an overall result, the encapsulation could be useful tool for the commercial sector when value added products are developed or in cases when product differentiation from competitors is achieved [5].

Encapsulation of therapeutics into liposomes represents a novel approach to sustained drug delivery [17]. Liposomes as drug carriers were first introduced around 1980 [18] and have been comprehensively

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investigated as carriers for the improved delivery of a broad spectrum of agents for more than 20 years [19]. They are lipid based microscopic vesicles that consist of an aqueous core entrapped by one or more bilayers composed of natural or synthetic lipids [17, 18]. Because of the ability to carry a broad range of substances, their structural diversity and their composition that makes them biocompatible and biodegradable, liposomes have been studied for many different therapeutic situations [18,19] and they represent one of the best colloidal drug carriers described by Bangham [17,20].

Possibility to adjust the drug release rate and the affinity for the target site by modifying the vesicular composition or surface properties, has made the development of liposomes as carriers to an ever-growing research area [21,18]. Due to the limited stability of liposomes during storage and administration [22], the research on liposome technology has progressed from conventional vesicles to “second-generation liposomes” in which liposomes with modified surfaces have also been developed using several molecules, such as surfactants [18]. Conventional liposomes are defined as vesicles which are commonly composed of solely phospholipids (neutral and/or negatively charged) and/or cholesterol [19]. Surfactants are indispensable as solubilizing agents in the isolation, purification and reconstruction of membranes. The interaction of surfactants with phospholipid membranes of liposome vesicles leads to different aggregated structures and ultimately to the formation of mixed micelles [23–26]. Phospholipid-detergent systems have been widely investigated, but it still remains undetermined how can the release of loaded therapeutics be affected by their encapsulation into the liposomes modified by surfactants [26]. For modification purposes, nonionic polysorbates (*i.e.*, Tween surfactants) are most frequently used [22,26,17].

The aim of this paper is to study diffusion coefficient and diffusion resistance.

Model of actives release from the carrier systems

As discussed, encapsulation of active substances is used to control their release [16] which is possible due to a fact that encapsulation will primarily lead to actives' sustained release caused by the decrease of the mass transfer coefficient and thus slower diffusion [27–31]. In order to characterize systems with sustained release, diffusion coefficient and diffusion resistance should be determined. The release of actives from various carriers could be treated as an unsteady diffusion process and their diffusion from a solution to another across a membrane in the standard Franz cell, could be approximated using Fick's second law and the following equation [27]:

$$\frac{c_{A,D} - c_{A,R}}{c_{A,D}^0 - c_{A,R}^0} = e^{-\beta Dt} \quad (1)$$

where $c_{A,D}$ and $c_{A,R}$ are concentrations of active in donor and receptor compartments at time t , and $c_{A,D}^0$ and $c_{A,R}^0$ are concentrations at time $t = 0$. D is the diffusion coefficient, t is time and β is the geometrical constant and represents characteristic of the particular geometry of the diffusion cell.

Mass transfer resistance (R) could be calculated as:

$$R = \frac{\delta}{D} \quad (2)$$

where δ is the sample thickness measured in the direction of mass transfer.

A serial diffusion resistance model could describe systems that include more than one diffusion resistance where the overall diffusion resistance represents the sum of individual resistances [27]:

$$R = \sum \frac{\delta_i}{D_i} \quad (3)$$

MATERIALS AND METHODS

Preparation of thyme extract

Thyme extract was prepared in compliance with the traditional aqueous extraction [14,15]. 10 g of dried thyme (Institute of Medicinal Plants Research Dr Josif Pancic, Serbia) was poured with 0.2 L of distilled water heated to 100 °C. The slurry was left to cool in a sealed glass beaker at room temperature, for 30 min. Supernatant solution was filtered.

Preparation of liposomes

Conventional liposomes, as well as liposomes with modified membrane, were prepared by the proliposome method [32]. The method is based on the initial formation of a proliposome mixture containing lipid, ethanol and water, which is converted to liposomes by a simple dilution step [32]. Phospholipon 90G (Phospholipid GmbH, Germany) was used as a membrane lipid. It comprises of phosphatidylcholine (min. 94 mass%) which is an unsaturated phospholipid and ensures liposomes to be highly bioavailable [33]. Ethanol was used as a lipid solvent and thyme extract as excess water.

Phospholipon 90G was measured at a glass beaker which was followed by the addition of the proper amounts of ethanol and water. The mixture was heated in a sealed beaker up to 60 °C with continuous stirring provided by the magnetic stirrer at 700 rpm. Stirring was applied at 60 °C until a homogenous dispersion was obtained. The composition was then cooled down to 25 °C. Afterwards, the liposomes were prepared by a

one-step addition of thyme extract into the lipid dispersion. Thyme extract was instilled whilst stirring at 700 rpm. The same stirring conditions were applied for additional 15 min. As a result, liposomal dispersion was obtained.

Preparation of modified liposomes was conducted following the same procedure, with the addition of corresponding Tween surfactant along with Phospholipon 90G. Compositions of liposomal dispersions for conventional liposomes, liposomes modified by Tween 20 and liposomes modified by Tween 60 are shown in Table 1.

Diffusion experiments

Diffusion of polyphenols through cellulose acetate membrane (pore size 0.20 μm) from thyme extract, liposomal dispersions of conventional liposomes, liposomes modified by Tween 20 and liposomes modified by Tween 60 was investigated. Experiments were conducted using the standard static Franz diffusion cell (PermeGear, Inc., USA) characterized by a 20 mL volume and diffusion area of 4.91 cm^2 [27].

Continuous stirring of the receptor fluid by a magnetic bar at 700 rpm was applied. The Franz diffusion cell was thermostated at 25 °C for 30 min, whilst donor and receptor chambers were charged with distilled water. Subsequently, the receptor chamber was filled with the receptor fluid – distilled water (25 °C). These were the initial steps for all of the diffusion experiments. The thyme extract was placed in the donor chamber which was then sealed with parafilm. Samples (0.5 mL) were taken from the receptor chamber and then compensated with the injection of the fresh receptor fluid (0.5 mL, 25 °C). Sampling was performed after designated time intervals: 15 min intervals for 2 h and then 30 min intervals until the experiment completion. Diffusion of polyphenols from thyme extract was observed for 3 h.

The same procedure was applied in all experiments and the liposomal dispersions of conventional liposomes, liposomes modified by Tween 20 and liposomes modified by Tween 60 were placed in the donor chamber. Diffusion of polyphenols from all liposomal dispersions was observed during 6 h. All experiments were replicated twice.

Determination of total polyphenol content (TP)

Total polyphenol content (TP) in thyme extract and samples collected during the diffusion experiments was spectrophotometrically determined using Folin–Ciocalteu's reagent, according to a modified method [34].

The procedure was as follows: 0.5 mL of the sample was pipetted into a 50 mL volumetric flask containing 30 mL of distilled water. Thereafter, 2.5 mL of Folin–Ciocalteu's reagent (Sigma-Aldrich, Germany) and 7.5 mL of 20% Na_2CO_3 were added. Content was mixed and the volume was made up with distilled water. After two hours the absorbance was measured by the UV spectrophotometer (UV/Vis Scanning Models/UV-3100 (PC), China) at 765 nm against a blank sample – distilled water. Gallic acid was used as the standard and the calibration curve was constructed with the results expressed as mg/L of gallic acid equivalents (GAE) [2].

RESULTS AND DISCUSSION

Concentration of polyphenols in the receptor chamber as a function of time, for all systems, was determined based on experimental data obtained from diffusion experiments. The results were then transformed into the dimensionless number, mass fraction $m_{R,t}/m_{D,0}$ and plotted against time as shown in Fig. 1a and b, where $m_{R,t}$ is the mass of polyphenols that was detected in the receptor chamber at a particular time and $m_{D,0}$ is the mass of polyphenols that was introduced into the donor chamber within the carrier system.

Figure 1a and b show that the vast accumulation of polyphenols in the receptor chamber was noted during the first 1.5, 2.5, 3.5 and 4 h for thyme extract, liposomal dispersions of conventional liposomes, liposomes modified by Tween 20 and liposomes modified by Tween 60, respectively. After these periods of time the diffusion of polyphenols gradually slows because the driving force is decreased. Therefore, the mass transfer is being reduced as the system approaches the steady state.

Plotted curves indicate that encapsulation of polyphenols in conventional and modified liposomes decreases the diffusion rates significantly. Comparison of the curves shows that the membrane modification of

Table 1. Compositions (g) of liposomal dispersions

Component	Conventional liposomes	Liposomes modified by Tween 20	Liposomes modified by Tween 60
Phospholipon 90G	0.50	0.89	0.89
Ethanol	0.50	2.00	1.00
Distilled water	1.00	1.00	2.00
Thyme extract	10.00	20.00	20.00
Tween 20	–	0.17	–
Tween 60	–	–	0.17

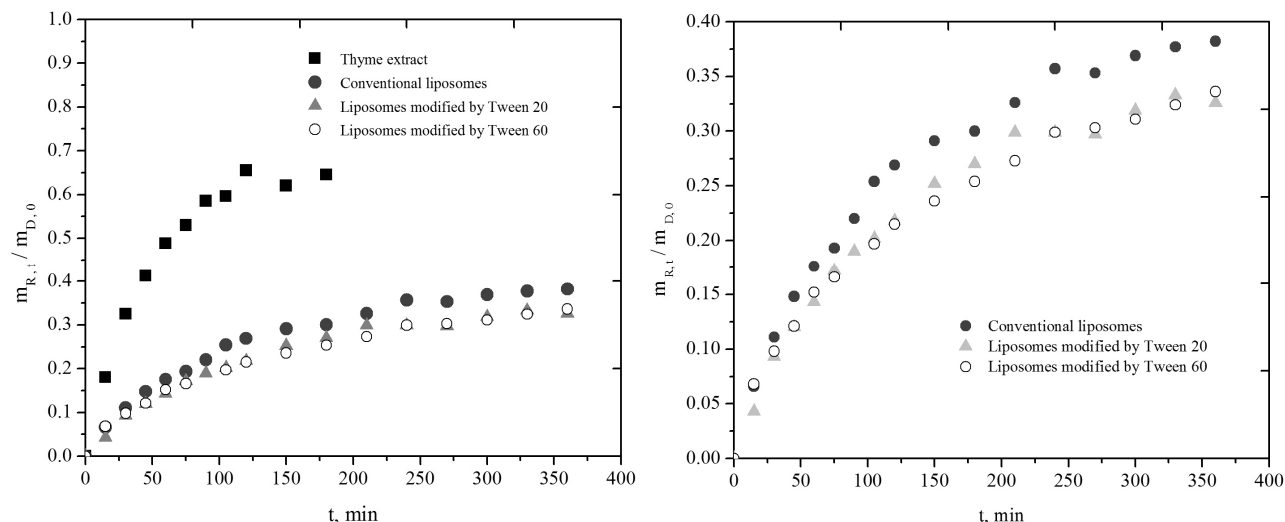


Figure 1. Released polyphenoles from thyme extract, conventional liposomes, liposomes modified by Tween 20 and liposomes modified by Tween 60 as a function of time.

liposomes with Tween 20 and Tween 60 additionally contributes to the decrease of the diffusion rate. In order to determine precisely the extent to which diffusion rate of polyphenols from modified liposomes is decreased, all experimental systems must be quantified. This is achieved by calculation and comparison of diffusion coefficients.

Diffusion coefficients of polyphenols from thyme extract and liposomal dispersions, across the membrane were calculated according to Eq. (1) from the slope of the linear part of curves:

$$\ln\left(\frac{C_D^0 - C_R^0}{C_D - C_R}\right)$$

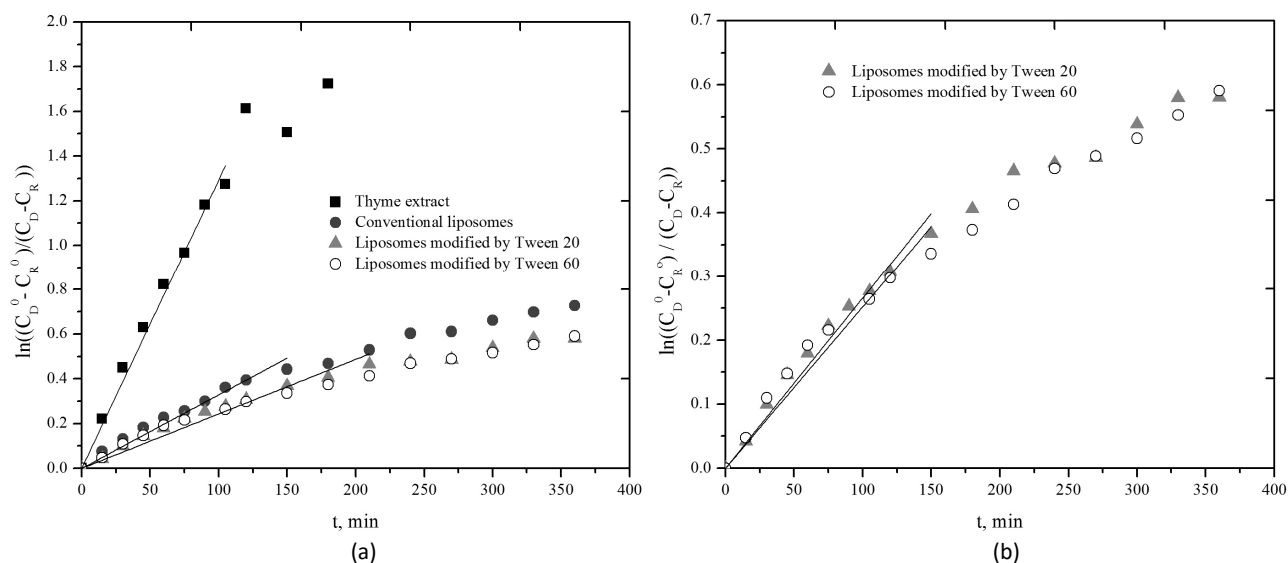


Figure 2. Dimensionless plot of polyphenoles concentration versus time for diffusion from investigated systems (a) and dispersions of modified liposomes (b).

versus time (Fig. 2a and b). β is the geometrical constant and represents characteristic of the particular geometry of the diffusion cell and membrane used in the experiments ($\beta = 2.49 \times 10^4 \text{ m}^{-2}$) [27]. Diffusion coefficient (D) of polyphenols from thyme extract is $8.77 \times 10^{-9} \text{ m}^2/\text{s}$, from liposomal dispersions are 2.31×10^{-9} , 1.78×10^{-9} and $1.69 \times 10^{-9} \text{ m}^2/\text{s}$ for conventional liposomes, liposomes modified by Tween 20 and liposomes modified by Tween 60, respectively.

Based on the values of diffusion resistances, it can be concluded to what extent encapsulation of polyphenols in liposomes contributes to the mass transfer decrease and how effective is the membrane modification. Known values of diffusion coefficients and sample thicknesses enable calculation of the diffusion resistances using Eq. (2). Since investigated systems

include two different diffusion resistances (the cellulose acetate membrane and the liposome particles), the serial diffusion resistance model can be applied as shown by Eq. (3). The diffusion resistance from thyme extract is the resistance that the cellulose acetate membrane provides diffusion of polyphenols. Overall diffusion resistances (R) are given in Table 2.

The diffusion resistance of liposomal membrane (R_{lip}) can be calculated as a difference between R values for specific liposomal dispersion and thyme extract. The results are shown in Table 3.

The results show that liposomes contribute to a higher overall diffusion resistance and that diffusion resistance of liposomal membrane is significantly higher when compared to the values obtained for system with unencapsulated polyphenols (thyme extract). Encapsulation of polyphenols into the conventional liposomes decreases the diffusion rate 3.80 times due to the liposomal membrane, which provides the additional diffusion resistance.

Table 2. Diffusion coefficients and overall diffusion resistances

System	$D/10^{-9} \text{ m}^2 \text{ s}^{-1}$	$R/10^6 \text{ s m}^{-1}$
Thyme extract	8.77	0.48
Liposomal dispersion – conventional liposomes	2.31	1.86
Liposomal dispersion – liposomes modified by Tween 20	1.78	2.37
Liposomal dispersion – liposomes modified by Tween 60	1.69	2.39

Liposomal membrane, which is modified by Tween 20, demonstrates further increase of diffusion resistance, thus slower diffusion rate which is 4.93 times slower, when compared with unencapsulated polyphenols. This type of modified liposomes also manifests different properties from the conventional liposomes. The permeability of the membrane with incorporated molecules of Tween 20 is lower than the one that is composed of phospholipids only, which is reflected by a fact that liposomes modified by Tween 20 show additional diffusion resistance in comparison with conventional liposomes.

Liposomes modified by Tween 60 exhibit similar features (Figure 2b). These particles show no significant difference in diffusion rates of polyphenols compared to liposomes modified by Tween 20. The results show that the diffusion rate is 4.98 times slower than in a system with unencapsulated polyphenols. The values of TP concentrations in the receptor chamber are very close to the ones obtained for system with liposomes modified by Tween 20. The explanation for this behavior could be found in a fact that both molecules Tween 20 and Tween 60 are polyoxyethylene (20) derivative of polysorbate, sorbitan monolaurate and sorbitan monostearate, respectively. The number 20

following the polyoxyethylene part refers to the total number of oxyethylene $-(\text{CH}_2\text{CH}_2\text{O})-$ groups found in the molecule. The number following the polysorbate part is related to the type of fatty acid associated with the polyoxyethylene sorbitan part of the molecule. Monolaurate is indicated by 20, monostearate is indicated by 60. It could be concluded from the experimental data that the diffusion of polyphenols from liposomes is not affected by the length of the fatty acid ester moiety, and mainly depends on the polyoxyethylene chain, and because of that, liposomes modified by both surfactants have similar diffusion resistance.

Table 3. Diffusion resistances of liposomes

Type of liposomes	$R_{lip}/10^6 \text{ s m}^{-1}$
Conventional liposomes	1.38
Liposomes modified by Tween 20	1.89
Liposomes modified by Tween 60	1.91

CONCLUSIONS

Liposome presence leads to a higher overall diffusion resistance compared to polyphenols diffusion resistance seen in thyme extract alone, and thereby promotes their prolonged release. Values of the diffusion resistance in liposome dispersions are about 4 times higher of those associated with unencapsulated polyphenols. This implies that liposomes are promising vehicles for protection and sustained release of polyphenols.

The liposome membrane modification can affect the rate of diffusion of the encapsulated polyphenols. Experimental results showed that the molecules of Tween 20 and Tween 60 further slow diffusion compared to conventional liposomes.

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IZVOD

UTICAJ SASTAVA MEMBRANE NA BRZINU OTPUŠTANJA POLIFENOLA IZ LIPIDNIH MIKROČESTICA

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(Naučni rad)

Sistemi za kontrolisano otpuštanje lekova predstavljaju istraživačku oblast koja se intenzivno proučava zbog niza prednosti koje ovi sistemi pružaju i mogućeg unapređenja dosadašnjih, tradicionalnih formi lekova. Fosfolipidne mikročestice (lipozomi) su se pokazale potencijalno pogodnim nosačima aktivnih supstanci u sistemima ovog tipa. Predmet ovog rada je ispitivanje uticaja sastava membrane lipozoma na brzinu oslobađanja inkapsuliranih polifenola. Eksperimentalna ispitivanja su pokazala kako modifikacija membrane lipozoma utiče na prenos mase, u odnosu na konvencionalne lipozome. Lipozomi su modifikovani površinski aktivnim materijama Tween 20 i Tween 60, dok je kao izvor polifenola korišćen čajni ekstrakt biljke majčine dušice. Eksperimenti su izvedeni korišćenjem Franz–ove difuzione ćelije, u kojima je praćena difuzija polifenola iz: čajnog ekstrakta, disperzije konvencionalnih lipozoma, disperzije lipozoma modifikovanih pomoću Tween 20 i disperzije lipozoma modifikovanih pomoću Tween 60. Obradom eksperimentalnih rezultata određeni su koeficijenti difuzije za svaki od sistema, kao i odgovarajući difuzioni otpori. Na osnovu dobijenih rezultata može se zaključiti da se inkapsulacijom polifenola u fosfolipidne mikročestice–lipozome, značajno usporava njihova difuzija, a da se modifikacijom membrane može postići dodatno usporavanje prenosa mase, što zavisi od strukture molekula kojim se modifikacija vrši. Molekuli Tween 20 kao i Tween 60 su se pokazali uspešnim modifikatorima membrane lipozoma. Otpori koje membrane modifikovane navedenim polisorbati pružaju difuziji polifenola su veći i dovode do 4,9 puta sporije difuzije u odnosu na sistem sa neinkapsuliranim polifenolima.

Ključne reči: Koeficijent difuzije • Difuzioni otpor • Lipozomi • Modifikacija membrane • Polisorbati • Polifenoli