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LIPOSOMES FROM HYDROGENATED SOYA LECITHIN FORMED IN SINTERED GLASS PORES

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Abstract: Possible complete closure of hydrophilic drug solutions in liposomes with required dimensions is the aim of variety liposome techniques. The ease of separating medication-loaded liposomes from liposome suspension to achieve an appropriate drug concentration in the final preparation is also desired. This paper describes the use of liposome preparation method, called reverse-phase evaporation, which leads to practical achievement of the earlier mentioned objectives. Preparation process is performed in an appropriately designed device. In optimal conditions of liposome preparation the final encapsulation efficiency of hydrophilic drug solution amounted to 50% in liposomes with a diameter in the range of a few micrometers up to 250 nm. The diameter of terminal liposomes is a simple function of relative amount of the lipid used and the degree of emulsion emulsification w/o at the beginning of liposome preparation. The density of the concentrated drug solution trapped in liposomes is usually higher than that of the buffer. Therefore, the loaded liposomes may be easily separated from non-trapped material by using of a simple sedimentation at 30000 × g. Density of aqueous drug solution insufficient to effective centrifugation can be magnified with an appropriate quantity of sucrose solution before encapsulation.

Keywords: encapsulation efficiency, reverse phase evaporation, emulsification, double emulsion

The liposomes are obtained with the use of many different methods, continuously improved in order to prepare homogenous and unimodal preparations (1). During their preparation, enclosing of aqueous solutions of drugs often takes place. Therefore, the preparation methods of liposomes have a major impact on their size, drug encapsulation efficiency (ee), lipid bilayer integrity, etc. The method based on vaporization of the hydrophobic solvent of lipids from w/o emulsion or double emulsion w/o/w, frequently modified, yielding liposomes called REVs (2). This method produces the liposomes of relatively high ee of an aqueous solution but they may contain too large quantity of residual hydrophobic solvent.

The aim of this work is to present the optimisation of REV method leading to a significant increase in ee of aqueous drug solutions in small liposomes. It can be achieved by preparing REV vesicles in the presented system. Essential elements of device were described previously (3). Scheme of the method described above was as follows. At the beginning, the w/o emulsion of aqueous solution to be entrapped (drug solution) in hydrophobic solution of lipids was formed. Afterwards, the w/o emulsion was dropped into the buffer and dispersed in order to form a double emulsion w/o/w but without mixing up both aqueous phases. Next, the double emulsion was repeatedly sucked with underpressure through a glass sinter with a simultaneous heating of the suspension. During sucking and heating the hydrophobic solvent was removed from the oil phase (in which phospholipids were diluted) and bilayers of vesicles were created. Introduction of intensive mechanical mixing w/o emulsion and an additional glass sinter G5 reduced the liposome size of 200–450 nm in diameter without ee decrease. Usually they were easily separated from suspension with centrifugation. In some cases, a small amount of sucrose in the trapped aqueous phase magnifies their density. It allowed vesicles separation from the suspension by simple sedimentation at 30000 × g.

At some quantitative proportion of lipid to drug to buffer volume during preparation, ee approximately 50% for many hydrophilic compounds to be entrapped were achieved.

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MATERIALS AND METHODS

Chemicals

Hydrogenated soy lecithin (HSPC) was a gift from Phospholipid GmbH (Cologne, Germany). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (DPPE) from Molecular Probes (Eugene, OR, USA) was used. Protoporphyrin IX disodium salt (PP) was from Sigma Chemical Co. (St. Louis, USA). Fluorescence label: N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (triethylammonium salt) (NBD-PE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) were from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). 4-Amino-10-methylfolic acid (MTX), Patent Blue V (PB), horseradish peroxidase (HP), cyclosporin A (CsA), cytochrome C (CytC) and PEG-2000 were from Fluka (Buchs, Switzerland). Organic solvents were from POCH (Gliwice, Poland). Chloroform and methylene chloride were distilled before use.

PEG-PE was synthesized by activation of PEG-2000 with disuccimidyl carbonate, followed by condensation with DPPE in chloroform in the presence of triethylamine (4).

The system for liposome preparation

The main components of the device depicted in Figure 1 was described previously (3). In comparison with the system described previously, the dropping funnel was removed and peristaltic pump was changed with HPLC pump [P]. During the whole procedure of the liposome preparation, the HPLC-pump pumps the suspension of vesicles (or buffer at the beginning of the vesicle preparation) from the bottom of the vessel [2] to the bottom of the central pipe [1]. It flows through the changeable glass sinter [4, G5] stopped with O-rings. On the bottom surface of the glass sinter [4] is located PVDF filter membrane 0.22 mm (Millipore Inc. USA) sealing in a casing with a silicone O-ring. The w/o emulsion of aqueous solution of the compound to be entrapped into vesicles and hydrophobic solution of lipids (in $CHCl_3/CH_2Cl_2$ 1:1 v/v) was produced outside in the special device by mechanical mixing at 11000 rpm (5). The ready w/o emulsion was gradually poured to the beaker [3] and further on mixed by magnetic stirring.

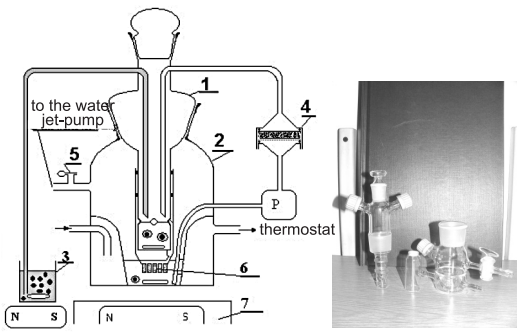

Figure 1. Scheme of the device for the preparation of vesicles in the glass sinter pores. [1] – central pipe with a flat bottom of inner diameter 12 mm, [2] – preparation vessel, [3] – the container of w/o emulsion, [4] – replaceable G5-glass sinter and membrane filter for calibration of vesicles, [5] – pressure reducing valve connected with water jet-pump, [6] – replaceable pipe with the glass sinter fused at the bottom, [7] – magnetic stirrer, [P] – HPLC pump, grey-organic solution of lipids, black circles – the drops of aqueous phase to be entrapped in vesicles. Photograph: central pipe [1] – on the left, pipe with the G1-glass sinter fused [6] in the middle, preparation vessel [2] on the right

The preparation of vesicles

At the outset of the preparation of liposomes, 6 mL of buffer, in which the vesicles would be suspended, was poured to the central pipe. From 2 to 50 mg HSPC was dissolved in 3 mL of CH_3Cl/CH_2Cl_2 (1:1 v/v) mixture. It was used to form w/o emulsion with 0.1–2.5 mL of aqueous solution (0.01 M TRIS-HCl buffered saline) of the hydrophilic compound to be entrapped into vesicles (for example PB, MTX, or other) as described above. After heating of the buffer (6 mL) in the vessel [2] to the desired temperature (45–55^{o}C), the pump P may be switched on. The buffer or double emulsion in the central pipe, as well as suspension in the vessel [2], was mixed independently with magnetic propellers. Both propellers were moved by a magnetic stirrer [7]. When the water-jet pump was running, from time to time, by pushing the valve [5], the excess of the double emulsion within the central pipe had to be sucked in through the glass sinter [6] to the vessel [2]. Then, w/o emulsion successively from the beaker [3] to central pipe [1] was sucked in self-actively. The double emulsion w/o/w was continuously pumped from the bottom of the vessel [2] to the central pipe through the glass sinter [4] and membrane filter. When the suspension in the vessel [2] was opalescent (usually 15–20 min after the w/o emulsion is dropped), i.e., hydrophobic solvent evaporated, the suspension of vesicles was ready.

The content of residual $CHCl_3$ within DPPC vesicles was estimated from ^{1}H NMR spectra (Bruker Avance 400 UltraShield spectrometer, Germany).

Conditions of the liposome preparation

The constant parameters of a single preparation were both the volume of the buffer (6 mL) in which

the resulting vesicles would be suspended and the volume of the organic solvent (3 mL). The quantity of lipids (from 2 to 50 mg) used in a single preparation and the volume of aqueous solution to be entrapped (from 0.1 to 2.5 mL) were the variable parameters of the liposome preparation. Hand-shaking or intensive mechanical mixing at 11000 rpm had got w/o emulsion (5). PB in buffered saline c = 20 mg/mL, MTX in 0.1 M NaOH c = 20 mg/mL, CytC c = 4 mg/mL + 0.1 ml saccharose c = 0.3 g/mL of 0.9% NaCl, HP c = 10 mg/mL 0.9% NaCl, CsA c = 10 mg/mL CH_2Cl_2, PpIX 10 mg/mL ethanolamine next diluted with 50 mL of 0.9% NaCl were encapsulated in liposomes.

Encapsulation efficiency

Within the vesicles, the hydrophilic compounds with a high- (CytC, HP) and low-molecular weight (PB, MTX, PpIX, CsA) were encapsulated. The resulting vesicles (6 – 8.5 mL) that encapsulated compounds with low-molecular weight were divided into two equal parts. The first was poured to the cellulose bag (Dialysis Tubing Cellulose Membrane, 32 × 31 mm, Sigma-Aldrich, Steinheim, Germany) and dialyzed over the buffer (100 mL) used in room temperature. After one day dialysis, the content of non-encapsulated compounds within the buffer was read spectrophotometrically (Spectrophotometer Jasco V-530, Osaka, Japan, PB at λ = 628 nm, MTX at λ = 370 nm, PpIX at λ = 407 nm). The results were compared with the diffusion data of a reference suspension of empty liposomes into which adequate quantity of aqueous solution of encapsulated compound (0.1–2.5 mL) was added. Cellulose bags were transferred to the fresh buffer every day during next 5–7 days and content PB, MTX or PpIX in dialyzates were read as above. From these data $t_{1/2}$ were determined. Second part was sediment at 30000×g × 15 min. Content of PB or MTX in supernatants was read spectrophotometrically as above. HP and CsA, non-loaded in liposomes, were separated by sedimentation only at above described conditions. HP content in supernatant was read spectrophotometrically (λ_{exc} = 280 nm, λ_{em} = 340 nm). CsA content in supernatant was read in ^{125}J automatic gamma counter WIZARD-1470 with the use of Cyclosporine direct RIA KIT (Immunotech, Prague, Czech Republic).

Liposome size

The size of liposomes was measured by dynamic light scattering with a size analyzer: Malvern Zeta Sizer 5000 (Malvern Instruments Ltd., Malvern, UK). Independently, the size and shape of the largest vesicles were estimated from images from an optical microscope Nicon-Eclipse EFD 3 (Japan).

Interior surface fraction of liposomes

Interior surface fraction *b* may be determined for empty vesicles labelled with NBD-PE and suspended in basic buffer as described previously (3). The assay allows differentiating unilamellar ($b \cong 0.5$) from multilamellar, multivesicular ($b > 0.5$) or leaky ($b < 0.5$) vesicles.

RESULTS AND DISCUSSION

The main idea of the method was a permanent separation of an aqueous solution of drug and the surrounding buffer by a hydrophobic solution of phospholipids (oil phase) in w/o/w double emulsion. Removing the hydrophobic solvents $CHCl_3$ and CH_2Cl_2 from the oil phase of w/o/w emulsion, but without infringement of their continuity, is a principal moment of an efficient encapsulation. Initially, a device in which w/o emulsion was sucked into the buffer with underpressure directly through the sinter [6] was tested. Suction through the sinter calibrates droplets of w/o emulsion in the buffer. It led, however, to fast filling of glass sintered pores with phospholipid. Continuous moistening of the sinter with buffer had eliminated this problem. The w/o emulsion is instilled to the layer of buffer or suspension at the bottom of the central pipe [2], but not directly on the sinter. Mixing this layer with magnetic stirrer created the double emulsion w/o/w. It may be sucked in through the sinter [6]. As sinter [6], the sinters of different density, from G1 to G5, were tested. The method requires a frequent sucking a double emulsion through the glass sinter with the use of underpressure. This was practically feasible for G1–G3 sinters only. Suction of w/o/w emulsion through the G4 and G5 sinters was ineffective. Additionally, at high underpressure, the double emulsion in vessel [2] was boiled and suspension was stretched from the vessel to the water jet-pump. During the creation of w/o emulsion, the aqueous phase was broken into drops of unknown dimensions, maybe with Gaussian distribution. They have steady tendency to join each other. Certain amount of lipid particles spontaneously locates itself on the border of the aqueous and the organic phase, while the rest was diluted in organic solvent. Hence, the quantity of lipid indispensable to the creation of a continuous lipid layer on the border of water and hydrophobic phase is dependent on size and number of drops of entrapped aqueous phase. The rest of the

Table 1. Encapsulation parameters of HSPC liposomes formed in sintered glass pores.

Lipid composition of vesicles[a]	Aqueous solution to be entrapped	Encapsulation efficiency (ee)[b] [%]	$t_{1/2}$[b] [days]	Temperature inside heating acket [°C]
HSPC (12)	PB	52 ± 4	45 ± 15	47
HSPC (6)	MTX	42 ± 3	55 ± 10	55
HSPC/DPPE-PEG [20:1 mol/mol] (4)	MTX*	53 ± 4	60 ± 15	47
HSPC (3)	CsA*	~100	–	55
HSPC (6)	HP*	46 ± 8	–	45
HSPC (7)	CytC*	45 ± 14**	–	55
HSPC (4)	PpIX*	~100	–	55

Aqueous solutions to be entrapped (0.5 mL) were dispersed within 3 mL of $CHCl_3/CH_2Cl_2$ (1:1 v/v) mixture that contains 10 mg HSPC. [a] number of preparations in parentheses; [b] the mean values ± SD; * solution to be entrapped was supplemented with sucrose, ** w/o emulsion obtained by hand shaking.

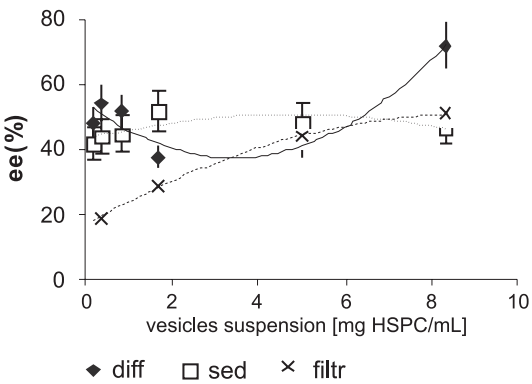

Figure 2. Encapsulation efficiency of PB solution in HSPC vesicles. Half milliliter of buffered PB solution (20 mg/mL) was entrapped in vesicles prepared from 2 to 50 mg HSPC with intensive stirring w/o emulsion. Final volume of vesicles suspension was equal to 6.5 mL. (diff) – ee data from diffusion data (G1+G5 used), (sed) – ee data from sedimentation data (G1+G5 used), (filt) – *ee* data from sedimentation data (G1+G5+membrane filter used)

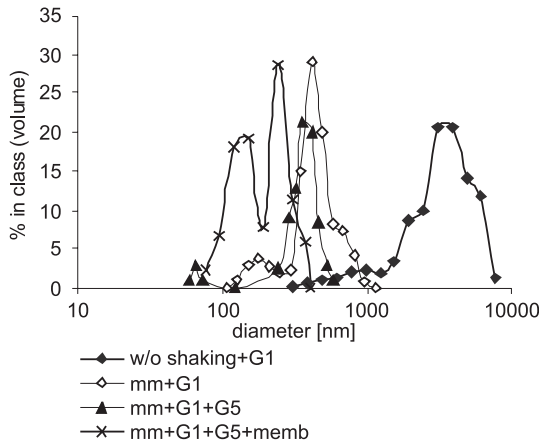

Figure 3. Size distributions of HSPC vesicles prepared with the different method of emulsion forming. The data were from Malvern Zeta Sizer 5000; (w/o shaking) – w/o emulsion obtained by hand-shaking aqueous and organic phase, G1, G5, memb – filter system used during vesicles preparation from 10 mg HSPC, as described in the text

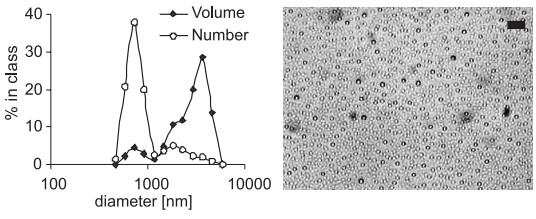

Figure 4. Number and volume distribution of vesicles prepared from 2 mg DPPC with the use of G1 filter only. Data from Mavern Zeta Sizer (on the left). Image from optical microscope (on the right). Bar indicates 10 mm

lipid should suffice to the creation of the outer lipid layer of a liposome bilayer, after the hydrophobic solvent is removed. For the creation of liposomes, one should remove the hydrophobic solvent that is contained in the oil phase, but without infringement of their continuity. The main part of encapsulated aqueous phase was located in sparse larger vesicles. Probably, in pores of G1 sinter, pre-liposomes undergo stretching that results in tearing them into smaller subunits. It reduced encapsulation efficiency

to about 50% (Table 1). Removal of glass sinter [6] from the device during preparation causes a fall of ee to about 20%. As can be seen, cyclosphorin A and protoporphyrin IX are located completely in hydrophobic core of HSPC bilayers. Encapsulation efficiency was correlated with quantity of HSPC and filter system used (Fig. 2).

As was ascertained, at quantitative proportion of drug solution to lipid solution to buffer equal to 1:6:12 (v/v/v), encapsulation efficiency was the highest one. The presence of a dense G5 sinter in the system lead to disruption of vesicles significantly larger than the diameter of sinter pores. Initial intensive mechanical mixing of w/o emulsion, which contain a sufficient amount of lipids, led to the formation of tiny droplets of water in w/o emulsion. They do not disrupt in G5 sinter and membrane filter. As can be seen in Figure 3, G5-filter alone i.e., without filter membrane was ineffective. Maybe suspension flows mainly through the space between G5 sinter and sinter chassis. For that reason the membrane filter was used.

A difficult problem to explain is the fact that liposomes prepared from the small quantity of lipid (1–2 mg) showed the largest ee ≈ 50% and high encapsulation volume. There is a simple approximate dependence between *ev* and average diameter *d* of formed vesicles: $d = 6 \cdot l \cdot \rho \cdot ev$, where: *l* is the thickness of lipid bilayer (~4 nm), ρ is the density of lipid bilayer (~1 g/cm^3). The data concerning ee (Fig. 4 on the left) suggested the formation also of large liposomes with diameter approximately 3–5 mm, however, liposomes of that size are not visible in the image from an optical microscope (Fig. 4, on the right).

^{1}H NMR 400 MHz spectra of pure PB, empty DPPC liposomes and DPPC liposomes loaded with PB (non trapped PB was removed with the use of centrifugation), all in D_2O/NaCl, showed distinct shifts ($\Delta\delta = \pm$ 0.01–0.09 ppm) in ^{1}H-resonances of PB benzene rings and of terminal methyl DPPC acyl chains. The glycerol ^{1}H-resonances of empty and loaded DPPC liposomes were identical (6). This suggests a meaningful superficial binding of PB particles to the DPPC layer (maybe instead of lipid particles). Detailed NMR analysis is not possible because CH_3-choline resonances overlap with CH_2 resonances of PB ethyl group, while CH_3- of PB ethyl groups overlap with $(CH_2)_n$ of DPPC multiplets.

^{1}H NMR measurements demonstrate $CHCl_3$ trace contents in the final suspension of DPPC liposomes, equal to 1.7 ± 1.0 ppm, read at chemical shift δ = 6.85 ppm in D_2O/NaCl.

Interior surface measurement of empty liposomes with the use of dithionite indicated that a hydrophobic solution of phospholipids in the fluid phase at room temperature (for example DOPC, SPC and DMPC) should not be mixed very vigorously with aqueous phase to be trapped in order to form w/o emulsion. In this case, gentle magnetic stirring was the best method to form the w/o emulsion. On the other hand, use of sonication or mechanical mixing results in a considerable decrease in encapsulation parameters and fraction of internal vesicles surface to $b \approx 0.1$–0.3 effective. Ultrasonication or intensive stirring was suitable to form w/o emulsion from hydrophobic solution of HSPC at room temperature.

Vesicles produced from relative large quantity of HSPC (30–50 mg) were oligolamellar or multivesicular because *b* values were in the range 0.6–0.7.

Summing up, in order to complete effectively aqueous phase in small unilamellar liposomes, it is necessary to disperse it completely in a hydrophobic lipid solution to droplets smaller than the diameter of membrane filter pores. In order to prepare a hydrophobic lipid solution, the amount of the lipid used should be at last sufficient to form complete lipid bilayers. As was affirmed, the optimum proportion of the substrates was 1 volume of encapsulated solution, 6 volumes of lipid organic solution and 12 volume units of buffer. The optimal lipid concentration was equal to 3–5 mg /mL of organic solvent.

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