LIPOSOMES FORMED IN SINTERED GLASS PORES

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Abstract: The method for preparation of vesicles, by evaporation of hydrophobic solvent from double emulsion (w/o/w) formed in the properly designed device is described. These method leads to multiple increase of encapsulation efficiency of aqueous solutions of drug in liposomes in comparison with other method. The w/o/w was passed through the glass sinter with the use of negative pressure to disrupt w/o/w drops into smaller ones. At low pressure and at higher temperature, the hydrophobic solvent from oil phase evaporated off and the lipids that were diluted in oil phase had created bilayer. When the relatively small quantity of lipids was used, the final encapsulation efficiency (ee) was about 50% and the uppermost encapsulation volume (ev) was 160 mL/g of lipids. Similar ee was noted for a 4-amino-10-methylfolic acid (MTX), Patent Blue V (PB) and bovine serum albumin (BSA). Liposomes loaded with drug at high concentration may be easily separated from suspension with the use of simple centrifugation.

Keywords: double emulsion, glass sinter(s), encapsulation, vesicles preparation

The vesicles may be obtained among other methods by vaporizing the hydrophobic solvent of lipids from the emulsion w/o or double emulsion w/o/w (1). The method gives liposomes named REV with relatively high encapsulation efficiency of aqueous solution of the order of 20-30 mL/g of lipid (2). Dehydration-rehydration or freezing and thawing of ready vesicle suspension enlarge an encapsulation efficiency of hydrophilic solution within liposomes (3). The high heterogeneity of vesicles diameter, may be reduced by multiple ultrafiltration of liposomes through adequate filters, however, with the reduction of encapsulation efficiency (4).

Modification of REV method leading to multiple increase of encapsulation efficiency of a hydrophilic drug(s) in liposomes is the aim of this work. It may be done in the properly designed device. The fundamental feature of the method is an essential increase of encapsulation efficiency of water phase within liposomes, quantitative transformation of used lipids into unilamellar liposomes and preparation of liposomes that may be separated from suspension by the use of simple centrifugation.

MATERIALS AND METHODS

Chemicals
Soy lecithin as L-α-phosphatidylycholine ≥ 99% (SPC) from ICN (Aurora, Ohio, USA) and hydrogenated soy lecithin (HSPC) from Phospholipid GmbH (Cologne, Germany) were used. 4-Amino-10-methylfolic acid (MTX), Patent Blue V (PB), bovine serum albumin (BSA) were from Fluka (Buchs, Switzerland). Fluorescence label N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (triethylammonium salt) (NBD-PE) was from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Sephacryl S1000 was obtained from Pharmacia-LKB (Uppsala, Sweden). Other reagents were from POCH (Gliwice, Poland). Chloroform and methylene chloride was distilled before use.

System for liposome preparation
The device depicted in Figure 1 is made from a glass mainly. The main part of the device is a central pipe (1) with a flat bottom and inner diameter 12 mm. Higher up the bottom (1 cm), there are four...
small wholes (diameter 1 mm) in the sidewall of the central pipe by which solutions from central pipe flow down on sinter (7). The other changeable pipe with the fused glass sinter (7, G1, G3 or G4 - the density of glass sinters) at the bottom end had been put through the two silicon O-rings on the bottom end of the central pipe. The lower space of the vessel (2) was heated through heating jacket with a hot water from the thermostat. The rubber tube connects the vessel (2) with the water-jet pump, through valve (5).

The P peristaltic pump (Pharmacia LKB, Uppsala, Sweden) pumps the suspension of vesicles or pre-liposomes or buffer at the beginning of the vesicle preparation from the bottom of the vessel (2) to the bottom of the central pipe via the PE-tube, during the whole procedure of liposomes preparation. At the outset of the vesicles preparation, 6 mL of buffer in which the vesicles would be suspended, was poured to the vessel (2) via central pipe, and heated to the desired temperature (30-65°C) through the water jacketed. The w/o emulsion of aqueous solution of the compound (from 0.5 to 2.5 mL) to be trapped into vesicles and hydrophobic solution of lipids (from 0.5 to 50 mg in 3 mL CHCl3/CH2Cl2 1:1 v/v) was produced outside in the beaker by mechanical stirring. The ready w/o emulsion was poured into the dropping funnel.

**Preparation of vesicles**

After the heating of the buffer in the vessel (2) to the desire temperature, the P pump was switched on. The w/o emulsion was dropped to the central pipe (1) in order to form a double emulsion (w/o/w). The buffer or double emulsion in the central pipe, as well as the suspension in the vessel (2) were mixed independently with magnetic propellers. Both propellers were moved with magnetic stirrer (8). When the water-jet pump is running, from time to time, by pushing the valve (5), an excess of the suspension w/o/w within the central pipe must be sucked in through the glass sinter (7) to the vessel (2). The double emulsion w/o/w is continuously being

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**Figure 1. Scheme of the device for preparations of vesicles using sintered glass filters. Real device is somewhat larger than on the sketch.**

(1) - central pipe with a flat bottom, (2) - preparation vessel, (3) - dropping funnel, (5) - pressure reducing valve connected with water-jet pump (6), (7) - the pipe with fused sintered glass filter, (8) - magnetic stirrer, (P) - peristaltic pump. Right scheme: Rectangles illustrate glass sinter (7). The w/o/w double emulsion flows down from the central pipe on a glass sinter (7). It is sucked with negative pressure to the vessel (2). (v) - finished vesicles, (grey) - hydrophobic solution of lipids or w/o emulsion, (black circles) - the drops of aqueous phase to be trapped in vesicles.
Liposomes formed in sintered glass pores

Table 1. Encapsulation parameters of liposomes formed in a glass sinter G1.

<table>
<thead>
<tr>
<th>Lipid composition of vesicles</th>
<th>Aqueous solution to be trapped</th>
<th>Encapsulation efficiency (ee) [%]</th>
<th>Encapsulation volume (ev) [mL/g]</th>
<th>Temperature inside heating jacket [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC</td>
<td>PB</td>
<td>58 ± 9**</td>
<td>190 ± 40</td>
<td>65</td>
</tr>
<tr>
<td>SPC</td>
<td>MTX</td>
<td>57 ± 7</td>
<td>185 ± 40</td>
<td>65</td>
</tr>
<tr>
<td>HSPC</td>
<td>PB</td>
<td>52 ± 4</td>
<td>160 ± 50</td>
<td>65</td>
</tr>
<tr>
<td>HSPC</td>
<td>BSA</td>
<td>49 ± 8</td>
<td>145 ± 35</td>
<td>55</td>
</tr>
</tbody>
</table>

*0.5 mL of aqueous solutions to be trapped were dispersed within 3 mL of CH3Cl/CH2Cl2 (1:1,v/v) mixture that contained 1 mg of lipids. The sedimented and next resuspended vesicles that captured PB solution had not shown its measurable release during 3-4 days of dialysis. ** ± S.D.

Figure 2. Size distributions of SPC vesicles prepared with the use of different glass sinters: G1, G3 and G4 at 30°C, the data from Malvern Zeta Sizer 5000. 1 mL of buffer was dispersed within solution of 1 mg SPC in 3mL of CH3Cl/CH2Cl2 (1:1 v/v) mixture. The dispersion was dropped into 6 mL of buffer and vesicles suspension was prepared as described above.

Encapsulation efficiency

The resulting vesicles (6.5-8.5 mL) that trapped compounds with low-molecular weight were transferred to the cellulose bag (Dialysis Tubing Cellulose Membrane, 32 × 31 mm, Sigma-Aldrich, Steinheim, Germany) and dialysed against the buffer (200 mL) used at room temperature. The content of non-encapsulated compounds within the buffer was read spectrophotometrically (Spectrophotometer Jasco V-530, Osaka, Japan). The results were compared with the diffusion data of a reference suspension of empty vesicles (6 mL) into which the aqueous solution of encapsulated compound (0.5-2.5 mL PB, or MTX solution) was added. Non-trapped BSA was removed from the vesicles on the Sephacryl-S1000 column (50 × 1,5 cm, Pharmacia LKB, Uppsala, Sweden), according to the suggestions of the gel supplier. The quantity of the non-trapped BSA was determined by Lowry method.

The size of liposomes was measured by dynamic light scattering with a size analyzer: Malvern Zeta Sizer 5000 (Malvern Instruments Ltd, Malvern, UK).

Interior surface fraction of vesicles

The assay allows differentiation of unilamellar from multilamellar or multivesicular vesicles labelled with NBD-PE. The changes of fluorescence emission intensity of NBD-label before and after the addition of sodium dithionite to the liposomes suspension were measured at \( \lambda_{em} = 535 \text{ nm} \) (exc = 463 nm) on SFM 25 KONTRON spectrophotometer (Kontron Instruments, Zürich, Switzerland) in accordance with the protocol described previously (5). Sodium dithionite reduced –NO2 group located in the outer surface of liposome bilayer to non-fluorescent –NH2 group. The fluorescence intensity after the reaction was normalized to the ratio of fluorescence before dithionite treatment, to obtain the percentage of fluorescence quenched by dithionite, i.e. the internal surface fraction.

RESULTS AND DISCUSSION

The main idea of the method was a permanent separation of aqueous solution of drug and surrounding buffer by hydrophobic solution of phospholipids (oil phase). Removal of hydrophobic solvent CHCl3 and CH2Cl2 from oil phase of w/o/w emulsion, but without infringement of theirs continuity, is a principal moment of the efficient encapsulation. Encapsulation efficiency has a weak corre-
lation with temperature, as was affirmed experimentally. Hydrophobic solvent was removed mainly when the pressure was reduced.

As can be seen in Table 1, encapsulation efficiency is about 50%, independently of lipid used and compound to be trapped. The diameter of w/o/w drops or liposomes is reduced during flows through the glass sinter. Contrary to expectations, the smaller vesicles, 100 – 300 nm in diameter, were produced with the use of G1 sinter i.e. with the largest pores (Figure 2.).

Probably, in G1 wide pores, they undergo larger stretching what results in tearing them onto smaller subunits. A difficult problem to explain was that liposomes prepared from the small quantity of lipid (1-2 mg) had revealed too large ee, about 50%, and high ev in respect to liposomes diameter. 1H NMR data had suggested meaningful superficial binding of hydrophilic PB to the bilayer, instead of lipid particles.

1H NMR measurements revealed trace amount of CHCl3, of the order to 1-2 ppm, in the final suspension of liposomes, read at chemical shift of 6.85 ppm in D2O/NaCl.

Summarizing, the described method permits to trap an aqueous solution of hydrophilic compounds in liposomes very effectively. Because the density of aqueous solutions trapped in liposomes was higher than of the buffer in which the liposomes were suspended, it permits to separate them from suspension with the use of simple laboratory centrifugation (20000 – 30000 × g).

REFERENCES