Chemistry and Physics of Lipids 164 (2011) 151–157

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Preparation of submicron liposomes exhibiting efficient entrapment of drugs by freeze-drying water-in-oil emulsions

Ting Wang[a,*], Ning Wang[a,*], Tongyan Wang[a], Weiran Sun[a], Tiefu Li[b]

[a] Department of Pharmacy, Jining Medical College, 669 Xueyuan Road, Sunshine City, Shandong Province 276826, China
[b] School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, Liaoning Province 110016, China

ARTICLE INFO

Article history:
Received 12 July 2010
Received in revised form 3 November 2010
Accepted 17 December 2010
Available online 24 December 2010

Keywords:
Phospholipid
Polyethylene glycol
Vesicle
Solid dispersion
Encapsulation
Particle size

ABSTRACT

A novel liposome preparation method is described as freeze-drying of water-in-oil emulsions containing sucrose in the aqueous phase (W) and phospholipids and poly(ethylene glycol)$_{1500}$ (PEG) in the oil phase (O). The water-in-oil emulsions were prepared by sonication and then lyophilized to obtain dry products. Upon rehydration, the dry products formed liposomes with a size smaller than 200 nm and an encapsulation efficiency (EE) higher than 60% for model drugs. The presence of lyoprotectant and PEG was found to be a prerequisite for the formation of liposomes with desirable properties, such as a small particle size and high EE. The lyophilates were stable and could be rehydrated to form liposomes without any change in size or EE even after a storage period of 6 months. Also, the lipophilic drug-containing FWE liposomes were stable and could be stored for at least 6 months although the liposomes containing hydrophilic drugs showed significant leakage. Based on the vesicle size and EEs of the model drugs, as well as the scanning electron micrograph (SEM) and small angle X-ray scattering (SAXS) pattern of the lyophilates, a possible mechanism for the liposome formation is proposed.

1. Introduction

Liposomes, vesicles enclosing an aqueous solution with bilayer membranes of phospholipids, have been extensively studied as microparticulate carriers for the efficient delivery of therapeutic agents since the first report by Bangham et al. (1965) (Fenske and Cullis, 2008). The superiority of liposomes as drug carriers is now widely recognized and, due to great advances in liposome research, some liposomal drugs have already been used clinically (Allen and Cullis, 2004; Fenske and Cullis, 2008). These advances include efficient liposome preparation methods, such as solvent injection, reverse-phase evaporation, freezing–thawing recycling, and dehydration–rehydration, as well as controlling liposome size by sonication or extrusion, lyophilization of liposomes, and, especially, two milestone techniques: the active loading of agents into liposomes and modifying the liposome surface using hydrophilic polymers, such as polyethylene glycol (PEG) and monosialoteteraahexosyl ganglioside (GM1) (Malam et al., 2009). Active loading, the remote loading of the agents into preformed liposomes using transmembrane ion gradients, was first proved valid by Deamer and coworkers in the mid-1970s (Deamer et al., 1972). However, it was a relatively long time before the method was recognized by Cullis and coworkers as a very efficient way to encapsulate amphiphilic therapeutic agents into liposomes (Mayer et al., 1985, 1986). However, using active loading method to prepare drug-containing liposomes usually involves several steps, such as the preparation of multilamellar vesicles (MLV) and subsequent large unilamellar vesicles (LUV), followed by establishment of a transmembrane ion gradient, and incubation to actively load drugs into liposomes (Fenske et al., 2003) and these steps are rather complex and time-consuming. Moreover, the method of active loading is only applicable to amphiphilic drugs; and other types of agents, hydrophilic and lipophilic ones, cannot be remotely loaded into liposomes. Although, up to now, a variety of other methods have been developed for the preparation of liposomes, but few methods can be conveniently used to prepare acceptable liposomal drugs (Barenholz, 2003; Fanciullino and Ciccolini, 2009; Kepczynski et al., 2008).

Recently, two novel methods based on freeze-drying of emulsions, namely freeze-drying of double emulsions (FDE) and freeze-drying of oil-in-water emulsions (FOE) (Wang et al., 2006; Wang et al., 2009b), have been proved valid for the preparation of submicron unilamellar liposomes. The EEs of the FDE liposomes are relatively high for lipophilic drugs but are still low (less than

Abbreviations: SPS, soybean phosphatidylserine; SPC, soybean phosphatidylcholine; PEG, poly(ethylene glycol)$_{1500}$; EE, encapsulation efficiency; CHO, cholesterol; SUC, sucrose; FLU, flurbiprofen; BER, berberin hydrochloride; PAE, paeoniflorin; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; FDE, freeze-drying of double emulsions; FOE, freeze-drying of oil-in-water emulsions; FWE, freeze-drying of water-in-oil emulsions; SSS, submicron spherical shell.

* Corresponding authors. Tel.: +86 633 2983690.
E-mail addresses: twangcn@yahoo.com (T. Wang), nwangcn@163.com (N. Wang).

0009-3084/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.chemphyslip.2010.12.005

20%) for the hydrophilic ones (Wang et al., 2006). The FOE procedure is simple, but without combination with remote loading, the prepared liposomes can entrap only a very small fraction (no more than 10%) of the non-lipophilic drugs (Wang et al., 2009b). Therefore, better methods for the preparation of liposomes with desirable properties, such as a small size and high EE, are still needed and being actively explored by researchers (Pereira-Lachataignerais et al., 2006; Zhigaltsev et al., 2005).

In this report a novel liposome preparation procedure is described, and it is similar to those of the FDE and FOE methods but involves freeze-drying of the water-in-oil (W/O) emulsions. This procedure includes preparation of W/O emulsions which contain in W sucrose as a lyoprotectant and in O phospholipids as an emulsifier and PEG as a solid dispersion matrix, lyophilization to remove solvents, and rehydration to obtain an aqueous dispersion of liposomes. This approach has been validated for the encapsulation of different kinds of agents (hydrophilic, amphiphilic, and lipophilic) into liposomes. Further investigation has confirmed that both the lyoprotectant and PEG are essential for the formation of liposomes with desirable properties, such as a small size with a narrow distribution and high EEs for model drugs.

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (SPC, Lipoid S 100, purity >94%, Lipoid, Ludwigshafen, Germany), soybean phosphatidylserine (SPS, Leci-PS90PN, purity >95%, Degussa, Freising, Germany), poly(ethylene glycol)$_{1500}$ (PEG, Sigma–Aldrich Co., Shanghai, China), cholesterol (CHO, purity >99%, Bodi Chemical Company, Tianjin, China), flurbiprofen (FLU, Keben Chemical Company, Hangzhou, China), paeoniflorin (PAE, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China), and berberin hydrochloride (BER, Xieli Pharmaceutical Co., Ltd., Pengzhou, China) were all purchased commercial products. The solvents used for the chromatographic mobile phases were of HPLC grade. Bidistilled water was prepared in our own laboratory and all other chemicals were of analytical reagent grade.

2.2. Preparation of liposomes by freeze-drying W/O emulsions (FWE)

The oil-in-water (W/O) emulsions were prepared by emulsification. A mixture of cyclohexane, dichloromethane and diethyl ether (4:1:1, v/v/v) was selectively used as the solvent for the oil phase (O), since the mixture has a high lipid-dissolving ability and its melting point is suitable for lyophilization. Bidistilled water was used as the solvent for the aqueous phase (W). Sucrose (SUC), used as a lyoprotectant, was dissolved in W to give a concentration of 5% (w/v). SPC, SPC/SPS (9:1, mass ratio), or SPC/CHO (4:1, mass ratio) was dissolved in O to give a lipid concentration of 15 mg/ml. PEG was also dissolved in O with a lipid/PEG mass ratio of 1:1. Three categories of model agents were used to be incorporated into liposomes: lipophilic flurbiprofen (FLU), dissolved in O with a drug/lipid mass ratio of 1:20; hydrophilic paeoniflorin (PAE) and amphiphilic berberin hydrochloride (BER), dissolved in W with a drug/lipid mass ratio of 1:1.

2.2.1. Formation of water-in-oil (W/O) emulsions

Firstly, 1 ml of W and 3 ml of O were added to a 10-ml ampoule. Then, using an ice/water bath to keep the temperature under 25 °C, the mixture was sonicated with a probe-type sonicator (JY92-II, Scientz Biotechnology Co., Ltd., Ningbo, China) in pulse mode (pulse on, 3 s; pulse off, 5 s; 80 W) to produce uniformly opalescent submicron W/O emulsions, which were immediately transferred to 5-ml freeze-drying vials with a fill volume of 1 ml, and rapidly frozen at −80 °C in an ultra cold freezer (MDF-U73 V, Sanyo Electric Co., Ltd., Japan).

2.2.2. Freeze-drying

This was carried out in a freeze-dryer (Eyela FDU-1100/DRC-1000, Tokyo Rikakikai, Japan) as follows: (1) freezing at −80 °C for 4 h (in an ultra low temperature freezer); (2) primary drying at −50 °C for 4 h, and at −37 °C, −20 °C for 2 h periods, respectively; and (3) secondary drying at 20 °C for 4 h. The chamber pressure was maintained below 5 Pa during the drying process. When the freeze-drying process was complete, the vials were immediately filled with nitrogen gas, sealed, and stored protected from light at 4 °C.

2.2.3. Rehydration

When needed, aqueous dispersions of liposomes were immediately formed by rehydration of the lyophilates with an aqueous solution that is isotonic with W at room temperature (above the phase transition temperatures, t_c, of the phospholipids used). The liposomes (or vesicles) prepared by the FWE procedure were called FWV.

2.3. Characterization of the FWE products

2.3.1. Dynamic light scattering (DLS)

The mean diameter (MD) of the FWV in aqueous solution was determined by DLS with a submicron particle size analyzer (LS230, Beckman Coulter Co., Fullerton, CA, USA) at room temperature. Each sample was measured three times and the results were given as an average MD ± SD (standard deviation), but the SD was the largest one of the three samples tested by DLS. The frequency size distribution with count basis was used for calculation of MD and SD. All calculations were performed using LS32 (version 3.19) software, and an optical model called psl.rfd was used.

2.3.2. Determination of EEs

For PAE and BER, the EEs were estimated from the following equation:

$$\mathrm{EE}\,(\%) = \frac{D_{\mathrm{tot}} - D_{\mathrm{free}}}{D_{\mathrm{tot}}} \times 100 \quad (1)$$

The free drug (D_{free}) was separated by ultrafiltration using a 10-ml stirred ultrafiltration cell (Amicon 8010, Millipore Corporation, Bedford, USA) fitted with a membrane with a cutoff of 10,000 Dalton. PAE and BER were assayed spectrophotometrically at UV detection wavelengths of 280 nm and 263 nm, respectively, using a double-beam UV–vis spectrophotometer (TU-1901, Purkinje General Instrument Co., Ltd., Beijing, China). The total content of drug (D_{tot}) in the preparations was determined after the liposome dispersion was diluted with an appropriate amount of methanol to disrupt the liposomes completely to release the encapsulated drugs into the solvent.

The EE for FLU was calculated based on the drug-to-lipid ratio of liposomes ($\mathrm{DLR}_{\mathrm{liposomes}}$) and the initial drug-to-lipid ratio ($\mathrm{DLR}_{\mathrm{initial}}$), and it was estimated from the following equation:

$$\mathrm{EE}\,(\%) = \frac{\mathrm{DLR}_{\mathrm{liposomes}}}{\mathrm{DLR}_{\mathrm{initial}}} \times 100\% \quad (2)$$

For the EE calculation, 100 μl aliquots of the liposome dispersion were placed on 1 ml Sephadex G-50 (medium) spin minicolumns to remove unencapsulated drug from the liposomes (Fenske et al., 2003). The amount of FLU in the excluded fraction was determined by HPLC (LC-6AD liquid chromatograph, SPD-20A UV detector, SIL-10AF autosampler, Shimadzu) at a UV detection