THE PREPARATION OF LIPOSOMES DERIVED FROM MIXED MICELLES OF LECITHIN ADDED BY SODIUM CHOLATE, FOLLOWED BY DIALYSING USING HEMOFLOW HIGH FLUX F60S

Erni H Purwaningsih[1], Rolf Schubert[2]

1. Department of Medical Pharmacy, Faculty of Medicine, University of Indonesia, Jakarta 10430, Indonesia
2. Institute of Pharmaceutical Technology, University of Freiburg, D-79104 Freiburg, Germany

Abstract

Liposomes are used for drug carriers meaning that drugs are incorporated in the membrane or the vesicle of the liposomes. In this study, liposomes were prepared from mixed micelles, consisting of phosphatidylcholine, without or with cholesterol and sodium cholate was added in several ratios namely 0.44; 0.55; 0.63; 0.70; 0.90 and 1.10. After the preparation, the sodium cholate has been removed by a dialysis membrane, using the Hemoflow High Flux, which is generally used for haemodialysis. The Hemoflow High Flux is a tool in an effort to obtain a simple, quick, effective method for removing sodium cholate in the process of preparing liposomes. The effectiveness of this tool was proved by the particle size of the liposome which was measured by the Malvern Particle Sizer. The particle size of the liposome consisting of phosphatidylcholine (PC) without cholesterol and with cholesterol was 63-68 nm at all ratios and approximately 125 nm at the ratio of 0.55; 0.63; 0.70, respectively. The particle size of the liposome tended to be smaller after dialyzing, although the concentration of lipids tended to increase. However, a large amount of buffer solution has to be used with this method.

Keywords: liposome, mixed-micelles, dialyzed membrane

1. Introduction

The activity of drugs depends partly on liposomes, drug vehicles or carriers, in which drugs are deposited in the membrane or vesicle of the liposomes. Several studies showed successful results in preparing the homogenous liposomes using detergents such as Triton-X100, bile salts etc.[1-4]. Sodium cholate was generally used to prepare liposomes with size ranging from 40-180 nm, depending on the ratio of lecithin and the composition of lipids. This technique used a large amount of buffer solution for the dialyzing of the detergent so that the main substance that should be incorporated into the liposome should have a hydrophilic character. This technique succeeded to entrap protein such as DNA or other genetic material which is useful for further research for gene therapy[5].

Among five established methods in preparing liposomes, one is the use of dialyzation systems after the mixed micelles of lecithin and detergent have been formed. In an effort to search for a rapid, simple and effective method to prepare liposome the Hemoflow High Flux, originally designed for haemodialysis was used in this study[1].

2. Method and Materials

Equipment: The Hemoflow High Flux (HHF), F60S, which has two surface membranes, an inner and an outer membrane, with a cut-off of 5000 Da. was donated by Uni-Klinikum, University of Freiburg. A spectrophotometer UNIKON, with a wave length of 830 nm, a Malvern Particle Sizer and two peristaltic pumps were used. The sample was pumped into the inner membrane of the cartridge of the HHF and the buffer was pumped into the outer membrane of the cartridge. Other equipments were Buchi Rotavapor-R, a water bath, and a vacuum pump.

Materials: High purity phosphatidylcholine from soyabeans (SPC) and egg yolk (EPC) were purchased from LIPOIDR. The sodium cholate detergent was from Sigma. Cholesterol was re-crystallized two times with methanol. Whatmann paper no. 1. Other chemicals were HEPES, phosphate buffered saline (PBS), demineralised water, polyethylene glycol (PEG) 4.000 and other additive chemicals.

Methods: the mixed micelles derived from both phosphatidylcholine, SPC or EPC and sodium cholate (SC) were prepared as follows:

- SPC or EPC (20 nm) and SC were weighed at molarity ratios of 0.40; 0.55; 0.63; 0.70; 0.90 and 1.10
- SPC or EPC with SC was mixed in a round bottom glass of 1 L.
- The mixture was dissolved in ethanol q.s. and evaporated by the Buchi Rotavapor-R with as rpm of rotation at point 6, the vacuum condition was started at 200m-Bar and decreased gradually to 10 m-Bar and the bottom of the container was emerged in a water bath at 400C. Then it was dry vacuumed at a high pressure, for 2-3 hours until the lipid film was completely dry.
- The next step was hydration with 250 ml PBS, pH 7.35 or with HEPES at pH 7.4, mixing thoroughly by handshaking and followed by magnetic stirring until an optimal clear solution was formed.
- Then it was dialyzed four times each with 500 ml buffer, passed through the outer membrane of the cartridge of HHF. The flow rate of the hydrated sample, passing through the inner membrane was 20 ml/min. and the buffer was 40 ml/min. The adjusted flow rate was higher than described in the literature[7], because in this study the flow did not have a constant rate below 20 ml/min. The volume of the liposome solution was increased to 700 ml because of this osmolarity.
- Finally the dialysate was concentrated using 2.5% of PEG 4.000 in 500 ml PBS. The volume of the liposome solution decreased from 700 ml to 150 ml. The concentration and the type of PEG were lower than described in the literature because at high concentration of PEG, the volume of the liposome solution decreased 12 minutes after dialyzing.
- The liposome size was measured, before and after every step of dialysis by Malvern Particle Sizer (MPS). The lipid concentration of those samples, which were hydrated with HEPES, was measured by Bartlett's method.
- The second experiment consisted of the liposome preparation containing cholesterol, for increasing the stability of EPC at a ratio of 1:3 and then it was mixed with sodium cholate at several molarities as described above. Lipids and sodium cholate were dissolved in methanol instead of ethanol, and the solvent was completely removed by Buchi rotavapor under reduced pressure.

Cholesterol was re-crystallized according to the following method:

- An amount of 4.85 g of cholesterol was dissolved into about 40 ml methanol using Buchi rotavapor, and the container was emerged in a water bath of 60^0C until all cholesterol was completely dissolved.
- The mixture was cooled at room temperature and 10-20% crystals were formed.
- Further it was cooled to 4^0C (the container should be emerged in ice or in an ice bag if necessary) until most of the material became crystals.
- The crystals, which were filtrated through papers, were dissolved again in methanol as described above. The crystals obtained then were dried by evaporating the remaining methanol in Buchi rotavapor at room temperature, followed by vacuum evaporation overnight.

3. Results and Discussion

All results are presented on tables. Table 1 shows the liposome size prepared from mixed micelle of SPC and SC at the ratio of 0.55 in PBS, pH 7.35. Sodium cholate (SC) was mixed using different methods: A. mixed in buffer (60.2-66.6 nm), B. mixed in lipid directly (52.2-62.7 nm). Table 2 displays the liposome size at other ratios i.e. 0.4-1.1, in HEPES buffer, pH 7.4. The size was almost similar at all ratios (54.4.7-80.8 nm). The liposome size of EPC, cholesterol and SC at several ratios in HEPES is shown in Table 3.The size after dialyzing (ratios 0.4-0.7) tends to be larger than a previous preparation i.e. 84.2-150.9 nm. At higher ratios, more than 0.7, the liposome size is too large. The hydrated lipid of PC (SPC or EPC) and sodium cholate at all ratios showed a clear solution after shaking thoroughly, but the mixture of EPC and cholesterol (3:1), remained cloudy in spite of overnight stirring, especially for the high rate ratio solutions of more than 0.63. The lipid concentration before and after concentrating with 2.5% PEG 4,000 is shown in Table 4. Before concentrating, the lipid concentration is about 17.6-24.2 mM and after concentrating is 21.1-39.4 mM. According to the theory, the concentration of lipids is 20 mM before preparation, however in this study amounts of lipids were lost or hyper-concentrated during preparation and dialyzing. A possibility which may caused this phenomenon is the width of the membrane surface of the cartridge (0.8-1.3 m^2) that allowed the lipid solution to pass through in or out the membrane during analysis[6,8].

Table 1.
The liposome size (nm) of SPC – sodium cholate (at the ratio of 0.55), before and after dialyzing with PBS, pH 7.35

	A	B
Before dialysis	*	*
After dialysis 1	66.6	*
After dialysis 2	60.2	52.2
After dialysis 3	66.3	60.9
After dialysis 4	65.2	62.7
After dialysis 5	63.9	62.5
Appearance of the solution	Clear	Clear

* undetectable because of the lowest intensity of the solution.

Table 2.
The liposome size (nm) of SPC and sodium cholate at several ratios, before and after dialyzing with HEPES buffer, pH 7.4

Ratio	0.44	0.55	0.70	0.90	1.10
Before dialysis	*	*	*	*	77.1
After dialysis 1	*	*	*	70.6	**
After dialysis 2	*	80.8	61.1	67.0	64.6
After dialysis 3	70.8	72.2	66.9	**	67.9
After dialysis 4	54.7	66.8	66.2	66.2	66.4
After dialysis 5	58.0	67.4	65.6	65.6	68.2
Appearance	clear	Clear	Clear	clear	cloudy

* undetectable. because of the lowest intensity of the solution.
** the sample was missing

Table 3.
The liposome size (nm) of EPC and cholesterol (3:1 mol/mol) and sodium cholate at several ratios, before and after dialyzing with HEPES buffer, pH 7.4

Ratio	0.4	0.55	0.63	0.7	0.9	1.1
BD.	153.0	138.7	240.5	191.9	183.5	175.9
AD.1	103.6	150.9	140.8	136.9	185.6	206.2
AD.2	65.9	145.2	127.1	129.3	139.3	361.9
AD.3	86.0	115.5	123.0	124.0	135.8	238.5
AD.4	84.2	123.3	121.3	145.1	134.9	362.3
AD.5	84.3	125.5	120.1	124.3	137.1	455.0
Appear*.	cloud	cloud	cloud	cloud	cloud	cloud

BD=before dialyzing; AD= after dialysing;
* After stirring for 2 h (at low ratio) and overnight (at high ratio, more than 0,63)

Table 4.
The lipid concentration (mM/L) before and after concentrating with 2.5% of PEG 4,000 at several ratios (PC:Chol=3:1) and sodium cholate*

Ratio lipid:detergent	Before concentrating (mM/L)	After concentrating (mM/L)
0.55	18.0 ; 17.6	22.9 ; 21.1
0.63	22.5 ; 20.8	39.4 ; 38.2
0.70	24.2 ; 23.3	32.6 ; 31.5
0.90	18.7 ; 18.2	25.9 ; 25.8
1.10	19.0 ; 18.3	25.9 ; 25.8

***** Measured by Bartlett's assay.

No statistically analyzed data are available at this stage, since not enough values could be compared. Detergents are widely used for the preparation of liposomes. An important parameter characterizing detergents and their suitability for this purpose is their critical micelle concentration (CMC) i.e. the concentration of monomers present in mixtures of detergents with water at concentration C more than CMC. Bile salts, especially sodium cholate, are naturally existing detergents with a rather high CMC of about 14-20mM[6,9], which are often used to produce uni-lamellar liposomes if combined with lipids. These are the best methods for preparing liposomes with lipophylic proteins inserted in the membranes, since these proteins can be introduced into the mixed micelles in the presence of mild, non denaturing detergents, to achieve a 100% incorporation rate without modification of the general method. Another special feature is the ability to vary the size of the liposomes by precise control of the condition of the detergent removal and to obtain vesicles of a very high homogenous size[6].

Depending on the lipid content and the lipid-detergent ratio, the size of the liposome could be defined. The particle size of about 70 nm from PC alone could usually be obtained at the molar ratio of 0.55 with sodium cholate[7,10]. In this study, at the same ratio, we obtained a particle size of 63-68 nm from SPC (Table 1 and 2). Our result seemed to be comparable with the result from other studies mentioned above, although a different method of dialysis was used. At the same ratio, the combination of PC and cholesterol (3:1 mol/mol) yielded with a size of 125 nm (Table 3). Another experiment using Mini-Lipoprep dialysator, using a membrane with a cut-off of 10.000, was carried out to remove the sodium cholate from the liposomes consisting of PC and cholesterol during 8 hours. A very homogenous liposome of 74 nm was produced. The different size of the produced substances may be caused by the different cut-off of the dialysator's membrane and the duration of the dialysis. In our study a membrane with a cut-off of 5000 with a duration time of about 2 hours was used.

The dialyzing method used in this study was found to be very effective in producing highly concentrated, sterile uni-lamellar liposomes in large amounts. However, some disadvantages were found. Only water soluble substances could be entrapped successfully into the vesicles, in addition with lipophilic proteins that could be incorporated in the membrane of vesicles. Also more than 2 hours is required to produce defined liposomes, which is considered to be time consuming and a large amount of buffer, 4-8 L is necessary to remove the detergent completely and to wash the dialysator finely[6]. The distribution of the particle size of our results (data is presented elsewhere) using the study method was very broad, perhaps because the diameter of the inner cartridge of the HHF was too large, about 200um, in order to be able to separate the small liposomes from the larger one. For homogenizing the particle size of liposomes, several methods should be adapted using gel chromatography with Sephadex G-50[6], Sepharose 2B-CL[11], or TLC with Sepharose4B[12].

With the study method, amounts of lipids, about 10%, were lost or hyper-concentrated during dialyzing. It is assumed that the loss of lipids was due to the fact that some of the lipids remained inserted on the surface of the membrane cartridge. The membranes have a wide size of 0.8-1.3 m^2 with a thickness of 11-40 um[6,8]. The existence of hyper-concentrated lipids was possible caused by the loss of the buffer solution after concentration with PEG 4,000. For the detection of the remaining lipids the washing solution of the cartridge should be further examined.

4. Conclussion

The method in this study for the preparation of liposomes could be used for mass production of liposomes, however it should be revised concerning the decrease of the amount of buffer solution during dialyzing. The particle size of the liposomes, which was 63-68 nm, tended to be stable at all ratios of the lipid detergents when preparing liposomes without cholesterol. The size of liposomes using cholesterol (approximately 125 nm), as a stabilizing membrane, tended to be higher than without cholesterol, and relative stable at ratio 0.55; 0.63 and 0.70. After dialyzing the concentration of the lipids tended to increase.

Acknowledgment

The authors would like to thank the URGE PROJECT badge III and IV, 1999-2000 sponsored by World Bank for funding this research. Our gratitude is extended to all staff members at the Institute of Pharmaceutical Technology and at Uni-Klinikum, University of Freiburg, and Prof. DR. DR. H-J Freisleben for their generous support during the study.

References

1. Lévy D, Bluzat A, Seigneuret M, Rigaud JL. A systematic study of liposome and proteoliposome reconstitution involving Bio-Bead-mediated Triton X-100 removal. *Biochim Biophys Acta* 1990; 1025: 179-190.
2. Schwendener RA, Asanger M, Weder HG. n-Alkyl-glucosides as detergents for the preparation of highly homogenous bilayer liposomes of variable sizes (60-240 nm diameter) applying defined rates of detergent removal by dialysis. *Biochem Biophys Res Com* 1981; 100(3): 1055-1061.
3. Lasic DD, Martin FJ. Phospholipid vesicles from mixed micelles of egg yolk lecithin and a zwitterionic detergent (CHAPS). *J Colloid Interface Sc* 1989; 133(2): 539-544.
4. Zumbuehl O, Weder HG. Liposomes of controllable size in the range of 40-180 nm by defined dialysis of lipid/detergent mixed micelles. *Biochim Biophys Acta* 1981; 640: 252-262.
5. New RRC. Preparation of liposomes. In: *Liposomes. A Practical Approach*. New RRC (editor). IRL Press at Oxford University Press, 1990: 33-104.
6. Goldin SM. Active transport of sodium and potassium ions by the sodium and potassium ion-activated adenosine triphosphatase from renal medulla. Reconstitution of the purified enzyme into a well defined in vitro transport system. *J Biol Chem* 1977; 252:5630-5642.
7. Hirnle P, Schubert R. Liposomes containing blue dye for preoperative lymph node staining: Distribution and stability in dogs after endolymphatic injection. *Internat J Pharmac* 1991; 72: 259-269.
8. Fresenius Polysulfone®.Hemoflow High-flux F60S.
9. Lasic DD. Chemistry of lipids and liposomes. *Liposomes from Physics to Applications*. In: Lasic DD (editor). Amsterdam: Elsevier Science Publisher BV, 1993: 14-34.
10. Rhoden V, Goldin SM. Formation of unilamellar lipid vesicles of controllable dimensions by detergent dialysis. *Am Chem Soc* 1979; 18 (19): 4173-4176.
11. Schubert R, Beyer K, Wolburg H, Schmidt KH. Structural changes in membranes of large unilamellar vesicles after binding of sodium cholate. *Biochem* 1986; 25: 5263-5269.
12. Van Renswoude AJBM, Blumenthal R, Weinstein JN. Thin layer chromatography with agarose gels. A quick simple method for evaluating liposome size. *Biochim Biophys Acta* 1980; 595: 151-162.