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REVIEW

LIPOSOMES PREPARATION METHODS

MOHAMMAD RIAZ
Faculty of Pharmacy, University of the Punjab,
Lahore, Pakistan

ABSTRACT

In this article information about various methods of liposome preparation (including the large scale manufacture) has been reviewed. The advantages and disadvantages of the methods have been described in terms of size distribution and encapsulation efficiency.

Liposomes are vesicles having concentric phospholipid bilayers (See Fig. 1). Molecules from low molecular weight (glucose) to high molecular weight (peptides and proteins) have been incorporated in liposomes. The water soluble compounds/drugs are present in aqueous compartments while lipid soluble compounds/drugs and amphiphilic compounds/drugs insert themselves in phospholipid bilayers. The liposomes containing drugs can be administrated by many routes (intravenous, oral inhalation, local application, ocular) and these can be used for the treatment of various diseases (Weinstein and Leserman, 1984).

A number of review article have been published about liposomes in general (Riaz et al., 1989) and about various aspects of liposomes -- the methods of preparation (Hauser, 1982), mechanism of liposomes fusion (Rand and Parsegiam, 1986), as drug carriers (Stuhne-Sekalec, 1991), temperature sensitive liposomes (Ozer et al., 1993), liposomes as topical drug carriers (Schrcir and Bouwslra, 1994), target sensitive liposomes (Hauying, 1994), pH sensitive I-liposomes (Chu and Szoka, 1994) and the stability and uses of liposomes (Riaz, 1995). In the present article, information about the preparation of liposomes have been reviewed.

Multilamellar liposomes (MLV) usually range from 500 to 10,000 nm. Unilamellar liposomes can be called as small (SUV) and as large (LUV); SUV are usually smaller than 50 nm and LUV are usually large than 50 nm. The liposomes of very large size are called giant liposomes (10,000 - 10,00,000 nm). They can be either unilamellar or multilamellar. The liposomes containing encapsulated vesicles are called multi-vesicular

liposomes. Their size range from 2,000-40,000 nm. LUVs having asymmetric distribution of phospholipids in the bilayers are called asymmetric liposomes.

Two parameters are used to describe the entrapment of water soluble compounds/drugs in the aqueous compartments of liposomes. The internal or trapped or captured volume is the volume enclosed by a given amount of lipid. It is expressed in µL/µmol or µL/mg of the lipid. The entrapment or encapsulation efficiency describes the percent of the aqueous phase (and hence the % of the water soluble drug) that be-comes entrapped during the liposomes preparation. It is expressed as % of original aqueous solution that was entrapped within liposomes of as % of the total material to be entrapped or as % entrapment/mg of lipid. The internal volume and encapsulation efficiency greatly depends on liposomal content, lipid concentration, method of preparation and the drug used. Table 1 gives the values of these two parameters for various types of liposomes. It should be noted that for hydrophobic compounds, entrapment efficiency is usually high irrespective of liposome type and lipid composition.

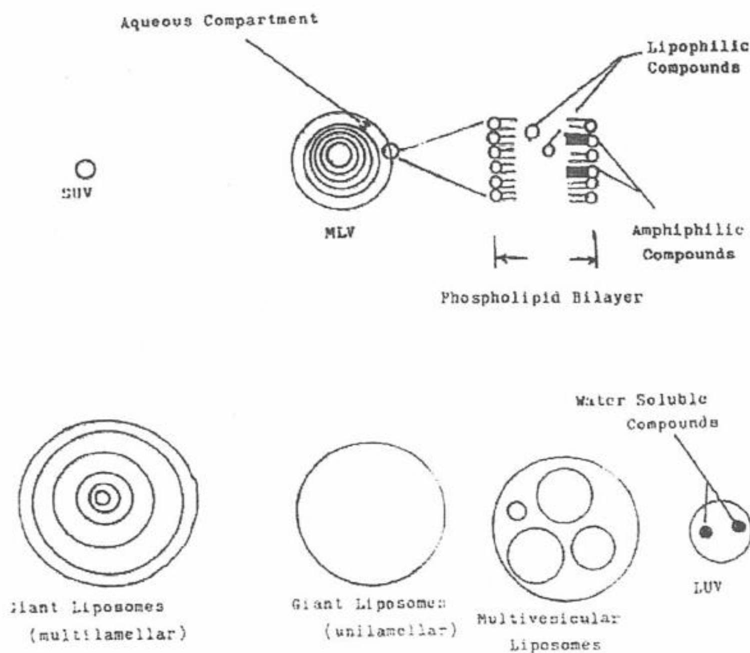

Fig. 1: Schematic represcmat ion of liposomc.
MLV = Mullilamellar liposomes;
LUV = Large unilamcllar liposomes;
SUV = Small unilamellar liposomes or vesicles.

Table 1
Internal volume and encapsulation efficiency of liposomes

Liposome Type			Internal Volume (μL/ μmole lipid)	(μL/ mg lipid)	Efficiency	Encapsulation Reference
MLV	(i)	Hydration Method*	1-4	--	10-25	Hauser, 1982
	(ii)	Solvent Spherule Method	0.3-2.7	0.5-4.0	0.3-1.6	Kim et al., 1985
SUV	(i)	Sonication Method	0.02-0.05	--	0.1-1.0	Hauser, 1982
	(ii)	French Pressure Method	0.2-1.5	--	--	Hauser, 1982
Multivesicular Liposome			10-79	15-127	11-89	Kim et al., 1983
Giant Liposomes			20	--	--	Oku et al., 1982
LUV	(i)	REV Method	--	0.5-15.6	35-65	Szoka and Papahadjopoulos, 1978
	(ii)	Modified REV Method	--	--	< 80	Handa et al., 1987
	(iii)	Freeze Thaw Method	< 10	--	20-30	Pick, 1981
	(iv)	Microfluidization Method	0.69-1.03	--	5-75	Mayhew et al., 1984
	(v)	Extrusion through polycarbonate filters under nitrogen	1.1-2.4	--	--	Hope et al., 1985

MLV = Multilamellar vesicles SUV = Small unilamellar vesicles
LUV = Large unilamellar vesicles REV = Reverse phase evaporation
*Hydration of lipids in the absence of an organic solvent.

Liposomes Preparation Methods

A) Multilamellar Liposomes (MLV)

(i) Lipid Hydration Method

(a) This is the most widely used method for the preparation of MLV. The method involves drying a solution of lipids so that a thin film is formed at the bottom of round bottom flask and then hydrating the film by adding aqueous buffer and vortexing the dispersion for some time. The hydration step is done at a temperature above the gel-liquid crystalline transition temperature Tc of the lipid or above the Tc of the highest melting component in the lipid mixture. The compounds to be encapsulated are added either to aqueous buffer or to organic solvent containing lipids depending upon their solubilities. MLV are simple to prepare by this method and a variety of substances can be encapsulated in these liposomes. The drawbacks of the method are low internal volume, low encapsulation efficiency and the size distribution is heterogeneous (Bangham et al., 1965, 1974).

(b) MLVs with high encapsulation efficiency can be prepared by hydrating the lipids in the presence of an immiscible organic solvent (petroleum ether, diethyl ether). The contents are emulsified by vigorous vortexing or sonication. The organic solvent is removed by passing a stream of nitrogen gas over the mixture. MLVs are formed immediately in the aqueous phase after the removal of organic solvent (Papahadjopoulos and Watkins, 1978; Gruner et al., 1985). The main drawback of this method is the exposure of the materials to be encapsulated to organic solvent and to sonication.

(ii) Solvent Spherule Method

A method for the preparation of MLVs of homogeneous size distribution was proposed by Kim et al. (1985). The process involved dispersing in aqueous solution the small spherules of volatile hydrophobic solvent in which lipids had been dissolved. MLVs were formed when controlled evaporation of organic solvent occurred in a water bath.

B) Small Unilamellar Liposomes (SUV)

(i) Sanitation Method

Here MLVs are sonicated either with a bath type sonicator or a probe sonicator under an inert atmosphere. The main drawbacks of this method are very low internal volume/encapsulation efficiency, possibly degradation of phospholipids and compounds to be encapsulated, exclusion of large molecules, metal contamination from probe tip and presence of MLV alongwith SUV. Recently, Oezden and Hasirci (1991) prepared a polymer coated liposomes by this method.

(ii) French Pressure Cell Method

The method involves the extrusion of MLV at 20,000 psi at 4°C through a small orifice. The method has several advantages over sonication method. The method is simple rapid, reproducible and involves gentle handling of unstable materials (Hamilton and Guo, 1984). The resulting liposomes are somewhat larger than sonicated SUVs. The drawbacks of the method are that the temperature is difficult to achieve and the working volumes are relatively small (about 50 mL maximum).

(iii) A new method for the preparation of SUV was given by Lasic et al. (1987). They deposited egg phosphatidylcholinc mixed with 1.5 %w/v of cetyl tetramethylammonium bromide (a detergent) in CHCI3/CH3OH on various supports for example silica gel powder, zeolite X, zeolite ZSM5. After the removal of organic phase, the system was resuspended by shaking or stirring in distilled water or 5 mM NaCl. There was some loss of phospholipid (about 10-20%) due to adsorption on the supports. The loss was 70% and 95% in the case of silica gel and zeolite ZSMS respectively. An homogenous population of vesicle with average diameter of 21.5 nm was obtained when zeolite X (particle size of 0.4 mm) was used as a support.

C) Large Unilamellar Liposomes (LUV)

They have high internal volume/encapsulation efficiency and are now a days being used for the encapsulation of drugs and macromolecules.

(i) Solvent Injection Methods

(a) Ether Infusion Method

A solution of lipids dissolved in diethyl ether or ether/methanol mixture is slowly injected to an aqueous solution of the material to be encapsulated at 55-65°C or under reduced pressure. The subsequent removal of ether under vacuum leads to the formation of liposomes. The main drawbacks of the method are that the population is heterogeneous (70-190 nm) and the exposure of compounds to be encapsulated to organic solvents or high temperature (Dcamcr and Bangham, 1976; Schieren et al., 1978).

(b) Ethanol Injection Method

A lipid solution of ethanol is rapidly injected to a vast excess of buffer. The MLVs are immediately formed. The drawbacks of the method are that the population is heterogeneous (30-110 nm), liposomes are very dilute, it is difficult to remove all ethanol because it forms azeotrope with water and the possibility of various biologically active macromolecules to inactivation in the presence of even low amounts of ethanol (Batzri and Korn, 1973).

(ii) Detergent Removal Methods

The detergents at their critical micelles concentrations have been used to solubilize lipids. As the detergent is removed the micelles become progressively richer in phospholipid and finally combine to form LUVs. The detergents were removed by dialysis (Kagawa and Rocker, 1971; Milsmann et al., 1978; Alpes et al., 1986). The advantages of detergent dialysis method are excellent reproducibility and production of liposome populations which are homogenous in size. The main drawback of the method is the retention of traces of detergent(s) within the liposomes. A commercial device called LIPOPREP (Diachema AG, Switzerland) which is a version of dialysis system is available for the removal of detergents. Other techniques have been used for the removal of detergents: (a) by using Gel Chromatography involving a column of Sephadex G-25 (Enoch and Suitt matter, 1979), (b) by adsorption or binding of Triton X-100 (a detergent) to Bio-Beads SM-2 (Gerristen et al., 1978). (c) by binding of octyl glucoside (a detergent) to Amberlite XAD-2 beads (Philippot et al., 1985).

(iii) Reserves Phase Evaporation Method

First water in oil emulsion is formed by brief sonication of a two phase system containing phospholipids in organic solvent (diethylether or isopropylether or mixture of isopropyl ether and chloroform) and aqueous buffer. The organic solvents are removed under reduced pressure, resulting in the formation of a viscous gel. The liposomes are formed when residual solvent is removed by continued rotary evaporation under reduced pressure. With this method high encapsulation efficiency up to 65% can be obtained in a medium of low ionic strength for example 0.01 M NaCl. The method has been used to encapsulate small, large and macromolecules. The main disadvantage of the method is the exposure of the materials to be encapsulated to organic solvents and to brief periods of sonication. These conditions may possibly result in the denaturation of some proteins or breakage of DNA strands (Szoka and Papahadjopoulos, 1978). We get a heterogeneous sized dispersion of vesicles by this method. Modified Reverse Phase Evaporation Method was presented by Handa et al. (1987) and the main advantage of the method is that the liposomes had high encapsulation efficiency (about 80%). The Reverse Phase Evaporation Method of Szoka and Papahadjopoulos (1978) has also been modified to entrap plasmids without damaging DNA strands (Haga and Yogi, 1989).

(iv) Calcium-Induced Fusion Method

This method is used to prepare LUV from acidic phospholipids. The procedure is based on the observation that calcium addition to SUV induces fusion and results in the formation of multilamellar structures in spiral configuration (Cochleate cylinders). The addition of EDTA to these preparations results in the formation of LUVs (Papahadjopoulos and Vail, 1978). The main advantage of this method is that macromolecules can he encapsulated under gentle conditions. The resulting liposomes are largely unilamellar, although of a heterogeneous size range. The chief disadvantage of this method is that LUVs can only be obtained from acidic phospholipids.

(v) Microfluldization Method

Mayhew et al. (1984) suggested a technique of microfluidization/microemulsification/homogenization for the large scale manufacture of liposomes. The reduction in the size range can be achieved by recycling of the sample. The process is reproducible and yields liposomes with good aqueous phase encapsulation. Riaz and Weiner (1995) prepared liposomes consisting of egg yolk, cholesterol and brain phosphatidylserin diasodium salt (57:33:10) by this method. First MLV were prepared by these were passed through a Microluidizer (Microlluidics Corporation, Newton, MA, USA) at 40 psi inlet air pressure. The size range was 150-160 nm after 25 recylces. In the Microluidizer, the interaction of fluid streams takes place at high velocities (pressures) in a precisely definedmicrochannels which are present in an interaction chamber. In the chamber pressure reaches up to 10,000 psi this can be cause partial degradation of lipids.

(vi) Extrusion under nitrogen through polycarboriatefilters LUV can be prepared by passing MLV under nitrogen through polycarbonate membrane filters (Jousma et al., 1987). The vesicles produced by this method has narrow size distribution. The extrusion is done under moderate pressures (100-250 psi). A special filter holder is required. Such devices are available commercially under the trade names such as LUVET and EXTRUDER and are equipped with a recirculation mechanism that permits multiple extrusion with little difficulty. Small quantities of liposome preparations (about 10 mL) can be easily prepared by the help of a commercial extruder. Riaz and Weiner (1994) prepared liposomes by this technique. The liposomes contained phosphatidylcholine from egg yolk and crude phosphoinositide sodium salt in the ratio of 4:1 and the lipid concentration was 12.5 /mole/ml. MLVs were passed through Extruder Lipex Membrane Inc., Vancouver, Canada) ten times through a stalk of two 100 nm polycarbonate filters (Nudeopore Pleasanton, CA, USA) employing nitrogen pressures upto 250 psi. Freeze fracture electron microscopy and p^{31}-FT NMR revealed that the liposomes were unilamellar. Photon Correlation Spectroscopy revealed that the size range was 99-135 nm.

(vii)Lasic et al. (1988) reported a method for the instant formation of a rather homogeneous preparation of LUV by a simple technique. The formation of multilammelar liposomes is prevented by inducing a surface charge (+ ve) on the bilayer while the size of the vesicles is controlled by the topography of the wafer support surface on which phospholipid film was formed. They deposited 0.5-1.0 mg egg yolk lecithin doped with 3 ml of $CHCl_3/CH_3OH$ on a specially ethched 2 inch silicon wafer. This wafer was put in place of the original bottom of an Erlenmeyer flask, that is bottom of the flask is replaced by wafer. After having dried overnight at 10^2 tore (about 1 Pa), the film was resuspended by gentle shaking in 1-2 ml water. Liposomes were formed instantly. The contamination of liposomes with large structures such as MLVs, giant vesicles and phospholipid particles was ruled out by video enhanced phase contrast microscopy.

(viii) A method for the extemporaneous preparation of LUVs has been described by Liautard and Phillippot (1993). The method was recommended for immediate clinical use of liposomes.

(ix) Freeze-Thaw Method

SUVs are rapidly frozen and followed by slow thawing. The brief sonication disperses aggregated materials to LUV. The formation of unilamellar vesiclesis due to the fusion of SUV during the processes of freezing and or thawing (Pick, 1981; Ohsawa et al., 1985; Liu and Yonethani, 1994). This type of fusion is strongly inhibited by increasing the ionic strength of the medium and by increasing the phospholipid concentration. The encapsulation efficiencies from 20 to 30% were obtained (Pick, 1981).

(D) Giant Liposomes

(i) The procedure for the formation of giant liposomes involves the dialysis, of a methanol solution of phosphatidylcholine in the presence of methylglucoside detergent against an aqueous solution containing up to 1 M NaCl. The liposomes range in diameter from 10 to 100 mm (Oku et al., 1982).

(ii) A method for the formation of giant single lamellar liposomes with size in the range of 10 to 20 prn by the removal of sodium trichloroacetate by dialysis was presented by Oku and MacDonald (1983).

(E) Multivesicular Liposomes

(i) The formation of multivesicular liposomes has been reported by Kim et al. (1983). The water in oil emulsion was converted to organic solvent spherules by the addition of the emulsion to across solution. The evaporation of organic solvent resulted in the formation of multivesicular vesicles. The diameter of liposomes ranges from 5.6 to 29 pm. The materials which can be encapsulated include glucose, EDTA, human DNA. These liposomes have very high encapsulation efficiency (up to 89%).

(ii) Cullis et al. (1987) found that when MLV preparations were subjected to five cycles of freeze On liquid nitrogen)-thaw and followed by thawing in warm water, the liposomes of high encapsulation efficiency (up to 88%) could be obtained. Freeze-fracture electron micrographs revealed vesicles within vesicles.

(F) Assymetric Liposomes

It has been shown that the phospholipid distribution in natural membranes is asymmetric. For example phosphatidytcholine and sphingomyelin concentrate at the

outer half of lipid bilayer whereas phosphatidylethanolamine, phosphatidylinositoland phosphatidylserine are mainly localized in the inner half of bilayer (Op den Kamp, 1979). Due to this, attempts have been made to prepare LUVs in which phospholipid distribution in both halves of bilayer is different. It appears that as model membranes the asymmetric liposomes are nearer to natural membranes than the conventional unilamellar liposomes. In the latter the phosphofpids distribution is symmetrical in bilayer.

(i)Cestaro et al., 1982 described a procedure for the preparation of asymmetric liposomes which contain cerebroside sulfate only at the outer leaflet of phospholipid bilayer. Cerebroside sulfate was adsorbed on to a filter paper (cellulose) support and then the support was incubated with small or large fused unilamellar liposomes. After six hours sulfatide contents reached about 6 mole percentage of the total quantity of phospholipid, corresponding to about 10 mole % of phospholipid present in the outer layer. The sulfatide could not be removed by washing with 1M NaCl or 1M urea.

(ii) Pagano et al. (1981) reported the formation of asymmetric phospholipid vesicles which contained fluorescent lipid analogue in either the outer or inner leaflet of the liposome bilayer. The procedure is based on the observation that the lipid analogues undergo rapid exchange (transfer) between the vesicles populations.

(iii) Denkins and Schroit (1986) prepared asymmetric liposomes by the enzymatic conversion of the fluorescent lipid-analogue of phosphatidylserine (NBD-PS) in the outer leaflet of LUV to NBD- phosphatidylethanolamine (NBD-PE).

(iv) Low and Zilversmit (1980) reported that lipid exchange proteins could be effectively be used so remove phosphatidylinositol at the outer leaflet of unilamellar liposomes. Therefore, it appears that these proteins may be used for the preparation of asymmetric liposomes.

(v) Collis et al. (1987) found that in SUV, distribution of lipid was not symmetrical and ratio of lipid in the outer monolayer to lipid in the inner monolayer could be as large as 2:1. Therefore, small unilamellar liposomes can be also be called as asymmetric to some extent.

INDUSTRIAL PRODUCTION OF LIPOSOMES

Of the several preparation methods described in the literature, only a few have potential for large scale manufacture of liposomes. The main issues faced to formulator and production supervisor are presence of organic solvent residues, physical and chemical stability, pyrogen control, sterility, size and size distribution and batch to batch reproducibility.

Liposomes for parenteral use should be sterile and pyrogen free. For animal experiments, adequate sterility can be achieved by the passage of liposomes through up to approximately 400 nm pore size Millipore filters. For human use, precautions for sterility must be taken during the entire preparation process: that is, (1) the raw materials must be sterile and pyrogen free, (2) preparation in sterile system: working areas equipped with laminar flow and (3) use of sterile containers (Freise, 1984).

Some issues related to phospholipids need attention. The liposomes based on crude egg yolk phospholipids are not very stable. The cost of purified lipids is very high. Recently, liposomes have been prepared using synthetic (Yamauchi et al., 1994) and polymerizable lipids (Fiona et al., 1987). The liposomes prepared from polymerizable phospholipids are exposed to UV light. The polymerization process takes place in the bilayer(s). Such liposome preparations usually have better storage stability. It should be noted that such materials usually are phospholipid analogues and their metabolic fates have yet to be established.

(i) Detergent Dialysis

A pilot plant under the trade name of LIPOPREP[R] II-CIS is available from Diachema, AG, Switzerland. The production capacity at higher lipid concentration (80 mg/ml) is 30 ml liposomes/minute. But when lipid concentration is 10-20 mg/ml 100 mg/ml then up to many litres of liposomes can be produced. In USA, LIPOPREP[R] is marketed by Dianorm-Geraete (Maierhofer, 1985).

(ii) Microlluidization

A method based on micro0uidization/microemulsiftcation/homogenization was developed for the preparation of liposomes. MICROFLUIDIZER[R] is available from Micro0udics Corporation, Massachusetts, USA. A plot plant based on this technology can produce about 20 gallon/minute of liposomes in 50-200 nm size range. The encapsulation efficiency up to 75% could be obtained (Mayhew et al, 1985).

(iii) Aqueous dispersions of liposomes often have tendency to aggregate or fuse and may he susceptible to hydrolysis and or oxidation. Two solutions have been proposed:

(iiia) Proliposomes

In proliposomes, lipid and drug are coated onto a soluble carrier to form free-flowing granular material which on hydration forms an isotonic liposomal suspension. The proliposome approach may provide an opportunity for cost-effective large scale manufacture of liposomes containing particularly lipophilic drugs (Payne et al., 1986).

(iiib) Lyaphilization

Freeze-drying (lyophilization) involves the removal of water from products in the frozen state at extremely low pressures. The process is generally used to dry products that

are thermolabile and would be destroyed by heat-drying. The technique has a great potential as a method to solve long term stability problems with respect to liposomal stability. It is exposed that leakage of entrapped materials may take place during the process of freeze- drying and on reconstitution. Recently, it was shown that liposomes when freeze-dried in the presence of adequate amounts of trehalose (a carbohydrate commonly found at high concentrations in organism) retained as much as 100% of their original contents. It shows that trehalose is an excellent cryoprotectant (freeze-protectant) for liposomes (Crowe et al., 1987). Freeze-driers range in size from small laboratory models to large industrial units are available from Pharmaceutical Equipment Suppliers. Recently Schrier et al. (1994) have studied the in vitro performance of formulations prepared from lyophilized liposomes.

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