
A Review on Formulation and Evaluation of Liposomal Drugs

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ABSTRACT: *A liposome is the drug delivery system used for the administration of various types of drugs or active substances essential for the treatment of various types of diseases. They play a major role in the target delivery of the drug to particular tissue without affecting the other body parts. Hence it is also called targeted drug delivery system. The term liposome means lipid body. It has been derived on the basis of name of subcellular particles, ribosome. Liposomes were first made by the British scientist Alec Bangham and colleagues at Babraham Cambridge in the mid- 1960s and they first published the structure of liposomes in 1964. Their size ranges from 25 to 500 nm. In this present review, we discussed about liposomes formulation, evaluation and their mechanism of action in the biological system.*

KEYWORDS: Liposomes, structural components of liposomes, Lipid excipients, method of preparation of liposomes, Evaluation of liposomes, marketed products.

INTRODUCTION

The name liposome is derived from two Greek words: Lipos = “fat” and Soma =”body”. Liposomes are simply vesicles or ‘bags’ in which an aqueous volume is entirely enclosed by a membrane composed of lipid (fat) molecules, usually natural or synthetic phospholipids. Liposomes are also defined as artificial microscopic vesicles consisting of aqueous compartment and surrounded by one or more concentric layers of phospholipids. The interior part of liposome consists of substances like proteins, hormones, enzymes, antibiotics, antifungal and anticancer

agents. Liposome is a small artificial vesicles of spherical shape that can create cholesterol and naturally non toxic phospholipid. They are depending upon size, hydrophobic and hydrophilic characteristic. Liposome is a spherical vesicles having at least one lipid bilayer¹⁻³.

It is used as vehicle for administration of nutrients as well as pharmaceutical drugs. It shows both characteristics-⁴

- 1) Hydrophilic head.
- 2) Lipophilic tail.

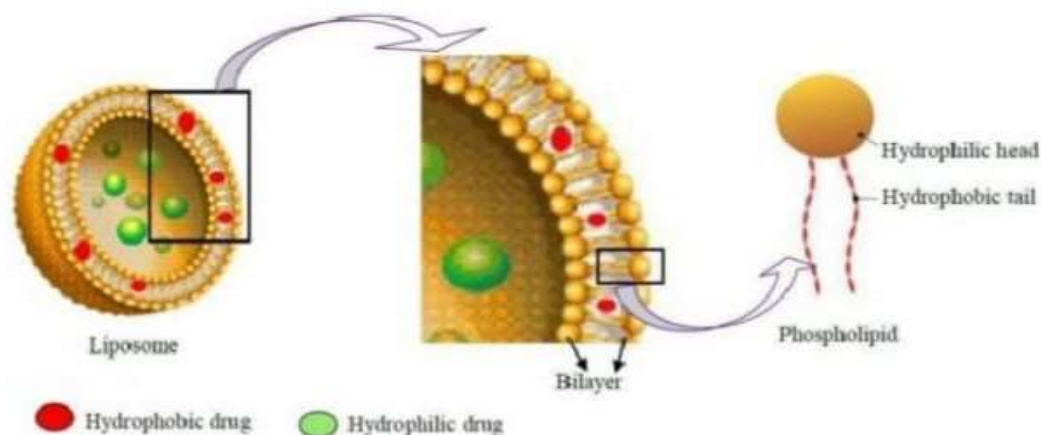


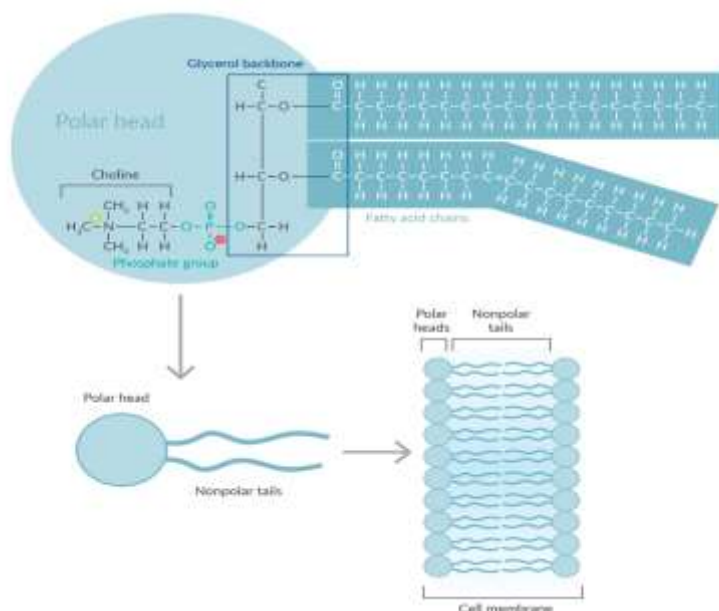
Figure-1 : structure of liposome.

Mechanism of liposome formation⁵:

- The liposomes are formed by hydrated phospholipids. So the physicochemical properties of phospholipids play a significant role in the liposome formation.
- Phospholipids are amphiphilic molecules (having affinity for both aqueous and polar moieties) as they have a hydrophobic tail is composed of two fatty acids containing 10-24 carbon atoms and 0-6 double bonds in each chain.
- In aqueous medium the phospholipids molecules are oriented in such a way that the polar portion of the molecule remains in contact with the polar environment and at the same shields the non- polar part.
- They align themselves closely in planar bilayer sheets to minimize the interaction between the bulky aqueous phase and long hydrocarbon fatty acyl chains.
- This alignment requires input of sufficient amount of energy (in the form of shaking, sonication, homogenization, heating, etc).

- Interactions are completely eliminated when these sheets fold over themselves to form closed, sealed and continuous bilayer vesicles.

Figure-2 : structure of a phospholipid.



Advantages of liposomes⁶⁻⁷

- Liposomes are biocompatible, completely biodegradable, non-toxic and non-immunogenic.
- Suitable for delivery of hydrophobic, amphipathic and hydrophilic drugs.
- Liposomes protect the encapsulated drug from the external environment.
- Reduce exposure of sensitive tissues to toxic drugs.
- These drugs provide controlled and sustained release.
- The drug can be stabilized from oxidation.
- Targetted Or site specific drug delivery.
- Control hydration.

Disadvantages of liposomes

- Production cost is high.
- Leakage and fusion of encapsulated drug/molecules.
- Short half-life.
- Stability problems due to flocculation.
- Allergic reactions may occur to various liposome constituents.
- Problem to targetting to various tissues due to their large size.
- Phospholipid may undergoes oxidation, hydrolysis.

Types of liposomes

Based on structural parameters

Unilamellar vesicle: Small unilamellar vesicles(SUV); Medium unilamellar vesicles(MUV); Large unilamellar vesicles (LUV)

Oligolamellar vesicles (OLV): These are made up of 2-10 bilayers of lipids surrounding a large internal volume.

Multilamellar vesicles (MLV) MLV made up of several bilayers. And the preparation method of MLV's differ from the other vesicle preparations. These MLV's arranged in an onion like manner in which concentric spherical bilayers of LUV/MLV enclosing a large number of SUV etc.

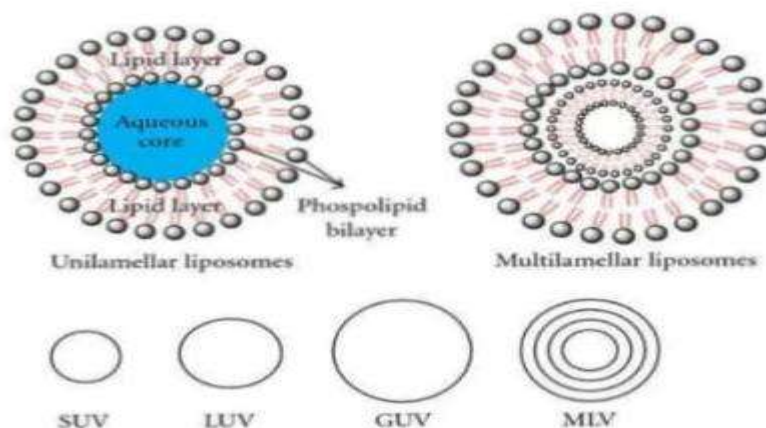


Figure-3. Different types of liposomes.

Based on method of preparation

1. Reverse phase evaporation method(REV) :single or oligolamellar vesicles.
2. MLV-REV:multilamellar vesicles made by a reverse-phase evaporation method.
3. SPLV: stable multilamellar vesicles.
4. FATMLV: frozen and thawed MLV.
5. VET: vesicles prepared by the extrusion method.

Based upon Composition and Application

- Conventional liposomes.
- Fusogenic liposomes(RSVE) :reconstituted sendai virus envelopes
- pH sensitive liposomes.
- Long circulatory liposomes.
- Immune-liposomes.

Classification of liposomes

Liposomes are classified into different types:

- According to their size.
- According to their number of lamellae (lipid bilayer).

Table 1 : Liposome Classification⁶

<i>Liposomes type</i>	<i>Size</i>	<i>No. of lamellae</i>
Small unilamellar vesicles(SUV)	20 nm-100 nm	single
Large unilamellar vesicles (LUV)	100 nm-400 nm	single
Glant unilamellar vesicles(GUV)	1-micro meter and large	single
Large multilamellar vesicles(MLV)	200 nm--~3micro meter	multiple
Multivesicular vesicles (MVV)	200 nm--~3micro meter	multiple

Composition of liposomes

The major structural components used in the preparation of liposomes are given below.

Phospholipids:- phospholipids are the main component for the preparation of liposome membrane. Most commonly used phospholipids in the liposome formulation are Glycerol containing phospholipids, as they represent greater than 50% of weight of lipid in biological membranes. These are derived from phosphatidic acid. The back bone of the molecule is glycerol moiety. And they are further categorized into two category:-

- Natural phospholipids:-
 - Egg phosphotidylcholine.
 - Soybean phosphotidylcholine
- Synthetic phospholipids:-
 - Dipalmitoyl phosphatidyl choline(DPPC).
 - Dipalmitoyl phosphatidyl ethanolamine(DP PE)
 - Distearoyl phosphatidylserine(DSPC).
 - Dipalmitoyl phosphatidic acid (DPPA).
 - Dioleoyl phosphatidylglycerol(DOPG).

Sphignolipids:-sphingosine is one of the most important parts of sphingolipids. Sphingolipids are obtained from plant and animal cells. This contains 3 characteristic building blocks.

- A mol of Fatty Acid
- A mol of sphingosine
- A head group that can vary from simple alcohols such as choline to very complex carbohydrates.

Eg:- Sphingomyelin and glycosphingolipids.

Gangliosides-found on grey matter, used as a minor component for liposome production. This molecule contain complex saccharides with one or more Sialicacid residues in their polar head group &thus have one or more negative charge at neutral pH. These are included in liposomes to provide a layer of surface charged group.

Cationic lipids

E. g. :DODAB/C-Dioctadecyl dimethyl ammonium bromide Or chloride.

DOTAP-Dioleoyl propyl trimethyl ammonium chloride-this is an analogue of DOTAP and various others including various analogues of DOTMA and cationic derivatives of cholesterol.

Sterols(Cholesterol)

Cholesterol and it's derivatives are often used in the preparation of liposomes, and it's help too-

- Reducing the permeability of the membrane to a water-soluble molecule.
- Decreasing the fluidity or microviscosity of the bilayer.
- Stabilizing the membrane in the presence of biological fluids such as plasma (this effect is used in the formulation of I. V. liposomes).

Polymeric materials

Synthetic phospholipids with diacetylenic group in the hydrocarbon chain polymerizes when exposed to U.V, leading to formation of polymerized liposomes having significantly higher permeability barriers to entrapped aqueous drugs.

E.g.: for other polymerisable lipids are- lipids containing conjugated diene, Methacrylate etc. Also several polymerisable surfactants are also synthesized.

Other substances

- Variety of other lipids of surfactants are used to form liposomes.
- Many single chain surfactants can form liposomes on mixing with cholesterol.
- Non ionic lipids
- A variety of Polyglycerol and Polyethoxylated mono and dialkyl amphiphiles used mainly in cosmetic preparations.
- Single and double chain lipids having fluoro carbon chains can form very stable liposomes
- Sterylamine and Dicapryl phosphate, incorporated into liposomes so as to impart either a negative or positive surface charge to these structures.

Preparation methods of liposomes⁴⁻⁹

Two methods are used for the preparation of liposomes. They are:-

- 1) General method of preparation.
- 2) A specific method of preparation:-these are mainly two types.

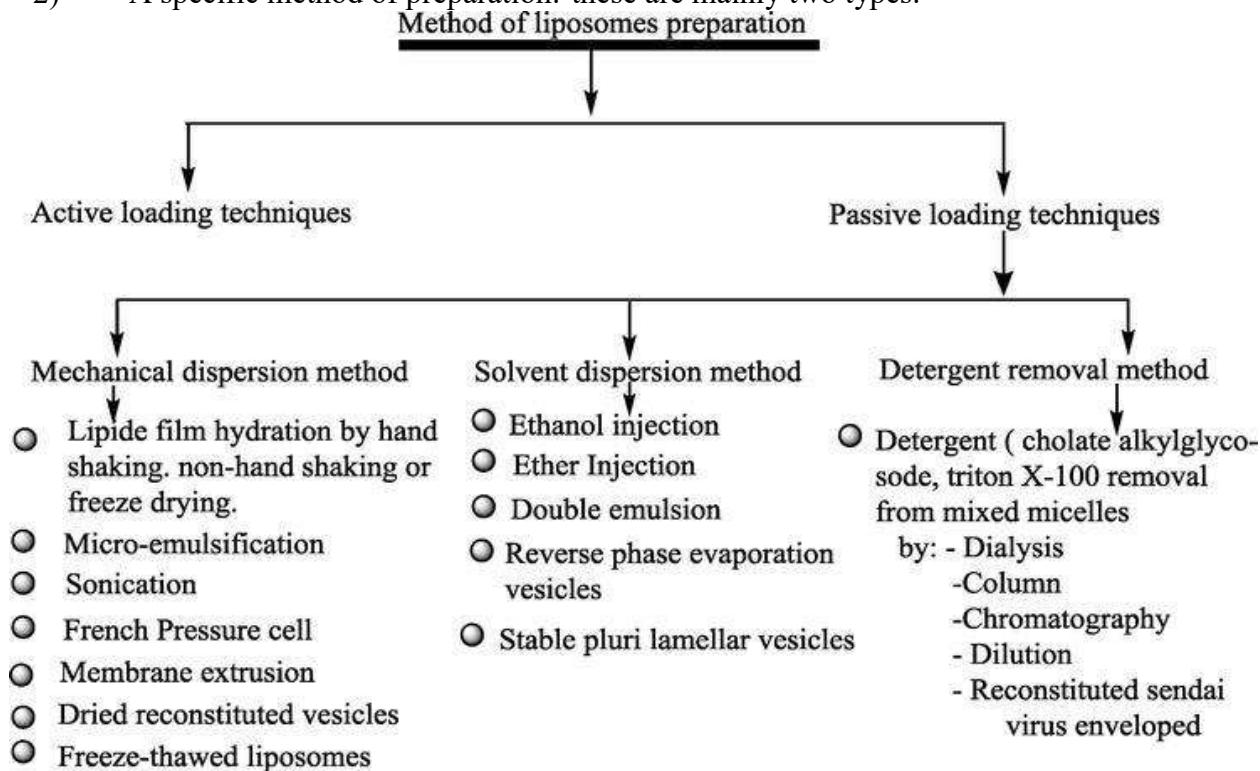


Figure 4: Methods used for the preparation of liposomes

1) **General method of liposome preparation:-**In all the methods which are used for the preparation of liposomes are involved in basic 4 stages are:-

➤ Drying down lipids from an organic solvent:-the lipid is dissolved in organic solvent. The solvent is evaporated leaving a small film of the lipids on the wall of the container. An aqueous solution of drug is added, the mixture is agitated to produce MLVs& then sonicated and the solvent is evaporated to get LUVs. After extrusion SUVs are formed.

➤ Dispersing the lipid in aqueous media:-drug can be incorporated into the aqueous solution or buffer, if it is water soluble (or) included in organic solvent, if it is hydrophobic.

➤ Purifying the final product:-free drug and liposomes can be separated by gel chromatography.

➤ Analyzing the final product.

2) **Specific methods of liposome preparation:-**

❖ Active loading technique:-

The active loading approach is also called remote drug loading, involves loading the drug agent after empty liposomes are produced. Liposomes are first generated containing a transmembrane gradient of pH or ion concentration is the the driving force to promote the drug diffuse across the membrane into the inner core of liposomes. i.e. aqueous phase inside and outside the liposome are different. Subsequently, an amphipathic drug is dissolved in exterior aqueous phase can permeate the phospholipid bilayer. After permeation interaction with trapping agent and core to lock- in the drug. The drug- entrapment process takes around 5-30mins, and a high loading efficiency (above 90%) can be reached.

In 1976, Deamer and Nicols demonstrate that a pH gradient could be utilized to load catecholamine into liposomes. Resulting stable drug retention in vitro.

❖ **Passive loading technique:-**Passive loading in which liposomes are formed concurrently with drug loading. In that hydrophilic compounds are distributed homogeneous in the aqueous phase (both inside and outside the liposomes), hydrophobic drugs are retaining inside the lipid bilayer of liposome, when working with water soluble drugs. The drug is firstly dissolved with lipid in organic solvent, followed by solvent evaporation method to prepare drug containing thin film. After prepare thin film hydrated with on aqueous phase to prepare liposome. When the loading of water soluble drugs, the film of lipid is dispersed in a drug-containing aqueous phase.

○ **Mechanical dispersion method:-**In these methods the aqueous volumes enclosed within lipid membranes is about 5- 10%, which is very small proportion of total volume used for preparation. So large amount of water soluble drug is wasted during preparation. But lipid soluble drug can be encapsulated to give 100% efficacy. In these methods, MLVs are formed and further treatment is required for preparation of Unilamellar vesicles.

● **Lipid hydration by hand shaking method:-**This is the simplest and widely used method. The lipid mixture of different phospholipids and charge components are dissolved in chloroform:methanol mixture (2:1v/v) and then this mixture is introduced in to a 250 ml round bottomed flask. The flask is attached to rotary evaporator connected with vacuum pump and rotated at 60 rpm. The organic solvents are evaporated at about 30 degrees. A dry residue is formed at the walls of the flask and rotation is continued for 15 minutes after dry residue appeared. The evaporator is detached from vacuum pump and nitrogen is introduced into it. The flask is then removed from evaporator and fixed onto lypholizer to remove residual solvent. Then the flask is again flushed with nitrogen and 5 ml of phosphate buffer is added. The flask is attached to evaporator again and rotated at about 60 rpm speed for 30 minutes or until all lipid has been removed from the wall of the flask. A milky white suspension is formed finally. The suspension is allowed to stand for 2 hours in order to complete swelling process to give MLVs.

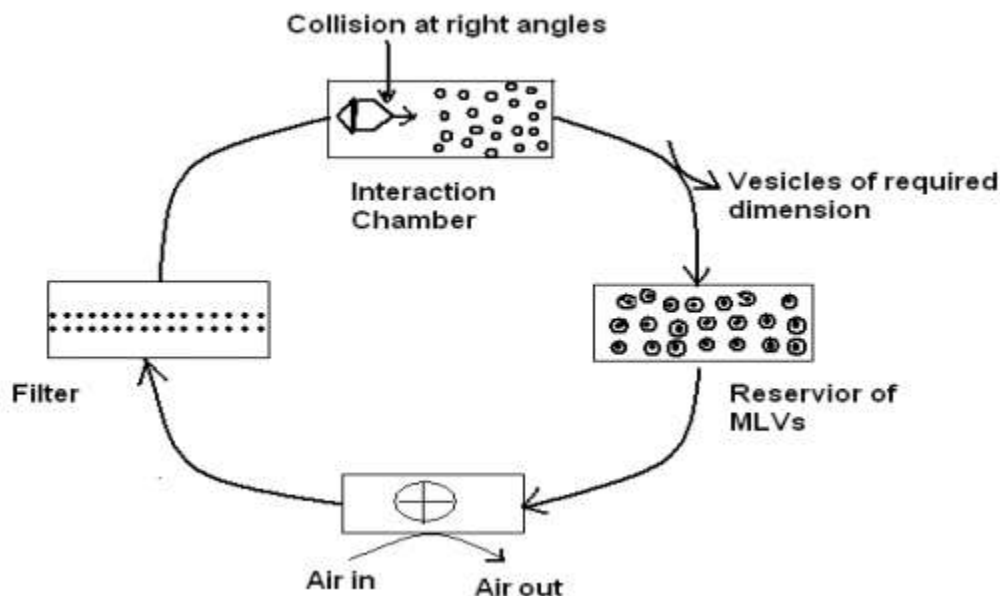
● **Non-hand Shaking method or freeze drying:-**

This is similar to shaking method except that care is taken in swelling procedure. The solution of lipid in chloroform and methanol mixture is spread over the flat bottom of the conical flask. The solution is evaporated at room temperature by flow of nitrogen through the flask without disturbing the solution. After drying water saturated nitrogen is passed through the flask until the opacity of

the dried film disappears. After hydration, lipid is swelled by addition of bulk liquid. The flask is inclined to one side, 10 to 20 ml of 0.2M sucrose in distilled water is introduced down the side of the flask and then flask is slowly returned to up right position. The solution is allowed to run gently over the lipid layer on the bottom of the flask. The flask is flushed with nitrogen sealed and allowed to stand for 2 hours at 37° for swelling. After that the vesicles are mixed to yield a milky suspension. The suspension is centrifuged at 1200 rpm for 10 minutes. The layer of MLVs floating on the surface is removed. From the remaining fluid, LUVs are produced.

- Micro-emulsification of liposomes:- From concentrated lipid suspension small vesicles are prepared by using an equipment called micro fluidizer. The lipids can be introduced into the fluidizer as a suspension of large MLVs. This equipment pumps the fluid at very high pressure through 5micrometer screen. Then it is forced long micro channels, which direct two streams of fluids collide together at right angles at very high velocity. (Speed of rotation is 20-200 rpm for biological). The fluid collected can be recycled through the pump and interaction chamber until spherical shaped vesicles are obtained.

Figure-4. Micro fluidizer.



- Sonication:- This method is used to reduce the size of the vesicles & imparts energy to lipid suspension. This can be achieved by exposing the MLV to ultrasonic radiation. There are 2 methods of sonication processes.

- Probe sonicator.
- Bath sonicator.

A) **Probe sonicator:-** probe sonicator is used for suspensions which require high energy in small volume.

Eg:- high concentration of lipids (or) viscous aqueous phase.

Procedure: The tip of sonicator is directly immersed into the liposome dispersion is very high in this method. The dissipation of energy at the tip results in local overheating. Then vessel must be immersed into an ice bath. Throughout, the sonication up to 1 hour more than 5% of the lipids can be de-esterify. Also, with the probe sonicator, titanium will slough off and contaminate the solution.

Advantages:

- Probe- sonication is commonly used to homogenize liposome formulations.
- It is necessary to investigate its influence on drug entrapment efficiency(EE) of liposome.
- By this method SUVs are formed and they can be purified by Ultra centrifugation.

Dis advantages:-

- The disadvantage of probe sonicator is contamination of preparation with metal from tip of probe.

B) **Bath sonicator:-** Bath sonicator is used for large volume of dilute lipids. .

Procedure :- The dispersion of liposome in a tube is placed into a bath sonicator. Controlling the temperature of the lipid dispersion. This method is easier to sonication the dispersion directly using tip. Material being sonicated and place into sterile container, under an inert atmosphere. Then lipid bilayer of the liposomes can fuse with other bilayers, thus delivering the liposome contents. By making liposomes in a solution of DNA or drug they can be deliver lipid bilayer.

Advantages:- Bath sonication is the most common instrumentation for preparation of SUVs.

- **French pressure cell method:-** This method is based on mechanism of high pressure. This method used to preparation of 1-40 ml of homogeneous unilamellar liposomes of intermediate size (30-80 nm). This liposome is more stable compared to the sonicated liposomes. This method has some drawbacks that are, initial high cost for the pressure cell. Liposome prepared by this method having less structural defects compared to sonicated liposome.
- **Membrane extrusion method:-** In this method the processed liposome has a narrow size distribution and selected average size less than about 0.4 microns.
- **Dried reconstituted vesicles:-** In this method, liposomes are added to an aqueous solution containing a drug or mixed with a lyophilized protein, followed by dehydration or mixture.
- **Freeze- thawed liposomes:-** In this method the SUVs are quickly solidified, followed by moderate defrosting.
- **Solvent dispersion :-** In these method can be dissolving the lipid and other constituents of the liposome membrane in other solution. The aqueous phase is added to resulting solution. In this aqueous phase contain material which is to be entrapped. Solvent dispersion method involving ether injection method, ethanol injection method, double emulsification method & reverse phase evaporation method.

- **Ether injection method:-** In this ether injection method, the solution of lipid is dissolved into ether or diethyl ether or methanol mixture. These mixtures slowly injected into aqueous solution of the material to be encapsulation at 55-65°C or under reduced pressure. Then ether is removed with the help of vacuum leads to formation of liposome.
- **Ethanol injection method:-** This is simple method. In this method an ethanol solution of the lipid is directly injected rapidly to an excess of saline through a fine needle. The solution of ethanol is diluted in water and phospholipid molecules. They are dispersed evenly through the medium. This procedure yields a high proportion of SUVs (about 25 nm diameter).

Figure-6: Ethanol injection and ether injection methods.

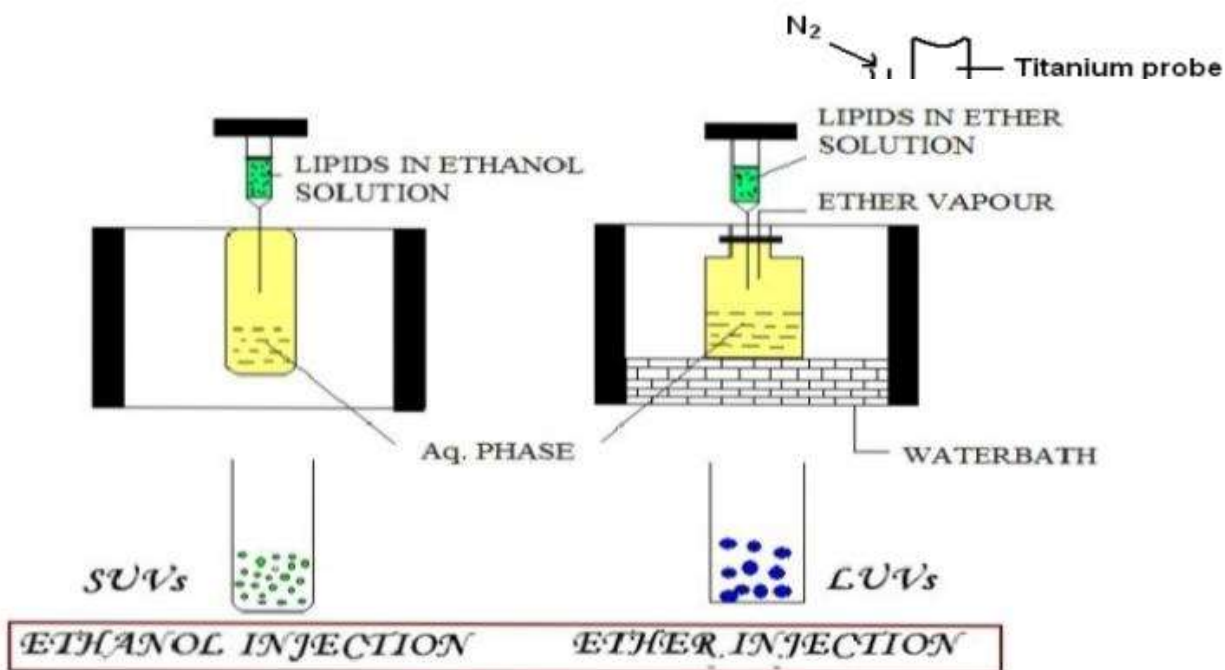


Figure-5: Bath sonication & probe sonication methods.

- **Double emulsion method:-** This technique is, also known as DepoFoamplatformTM, has been adopted by three commercial products of DepoCyte, DepoDur, and Expel to produce MVLs. The whole production routinely includes four sequential operations as follows:

- 1) the formation of a “water-in-oil” emulsion,
- (2) the formation of a “water-in-oil-in-water” emulsion
- , (3) solvent extraction with the help of stripping gas or vacuum pressure, and
- (4) microfiltration for the removal of the free drug, concentration, and exchange of external solution.

- **Reverse phase evaporation vesicles :-** The water in oil type of emulsion is formed by sonication of two phase system. It contains phospholipid in organic solvent (diethyl ether)& aqueous buffer. This mixture is added to round bottom flask. Under certain pressure by using rotary

evaporator the organic solvent is removed. This system is purge with nitrogen and lipids, are re-dissolved in the organic phase. Diethyl ether & isopropyl ether are the solvent of choice after the lipids are re-dissolved the emulsion are obtaining & then the solvents are evaporated by evaporation of semisolid gel under reduced pressure, at 20-25°C rotating at approximately 200rpm. A viscous gel forms and an aqueous suspension appears. Add excess water or buffer and evaporate the suspension for an addition 15 minute at 20°C to remove traces of solvent. Dialyze the preparation, and pass through 4B column or centrifuge. Resulting liposome are called 'reverse phase evaporation vesicle' (REV).

- **Stable plurilamellar vesicles:-** It involves preparation of water in organic phase dispersion with an excess of lipid followed by drying under continued bath sonication with stream of nitrogen. The internal SPLV is different from that of MLV-REVs, in that they lack a large aqueous core. The internal environment of both the vesicle is different from each other.

- Detergent removal technique:-

- **Detergent (cholate, alkyl glycoside, Triton X-100) removal of mixed micelles (absorption)**

Detergent absorption is attained by shaking of mixed micelle solution with beaded organic polystyrene absorbers such as XAD-2 beads (SERVA Electrophoresis GmbH, Heidelberg, Germany) and Bio-beads SM2 (Bio-Rad Laboratories, Inc., Hercules, USA). The benefit of detergent absorber is removal of detergent at very low CMC.

- **Dialysis :-** The detergent at their critical micellar concentration (CMC) is used to solubilize lipids. The detergent is detached, the micelles in phospholipid and last combine to form LUVs. The detergent can be removed by dialysis.

Advantages: Benefit of detergent dialysis method is formation of liposome populations which are homogeneous in size.

Disadvantages:- The main disadvantage of this method is possibility retention of traces of detergents into the liposome.

- **Dilution:-** The dilution of aqueous mixed micellar solution of detergent and phospholipids with buffer. The size of micellar and polydispersity is fundamentally increase.

Characterization of liposomes:- Liposomal processing and formulations for specified purposes are characterized to ensure their predictable in vivo and in vitro performance. The characterization parameters for purpose of evaluation could be classified into three categories.

- 1) Physical characterization.
- 2) Chemical characterization.
- 3) Biological characterization.

- 1) **Physical characterization:-** various parameters has been evaluated in the physical characterization of liposomes. Those are: size, shape, surface features, release profile and phase behaviour.

- 2) **Chemical characterization :-** It includes study of purity and potency of various lipophilic constituents.

3) **Biological characterization:-** To evaluate the safety and suitability of the formulation for therapeutic application.

Table 2 : Liposome characterization.

Characterization parameters	Instrument for analysis
Vesicle shape and surface morphology	TEM and SEM
Vesicle size and size distribution	Dynamic light scattering TEM
Electrical surface potential and surface pH	Zeta potential measurement and pH sensitive probes.
Surface charge	Free flow electrophoresis
Lamellarity	P31 NMR
Phase behavior	DSC, Freeze fraction electron microscopy
Percent capture	Mini column centrifugation
Drug release	Diffusion cell/ dialysis

Chemical characterization

Characterization parameters	Instrument for analysis
Phospholipids concentration	HPLC/ Barret assay
Cholesterol concentration	HPLC/ Cholesterol oxide assay
Phospholipids per oxidation	U.V Observation
pH	pH meter
Osmolarity	Osmometer

Biological characterization

Characterization parameters	Instrument for analysis
Sterility	Aerobic/ Anaerobic culture
Pyrogenicity	Rabbit fever response
Animal toxicity	Monitoring survival rats.

Therapeutic applications of liposomes:-

- Liposomes as drug / protein delivery vehicles Controlled and sustain release in situ.
- Enhanced drug solubilization .
- Enzyme replacement therapy and lysosomal storage disorders.
- Altered pharmacokinetics and biodistribution.
- Liposomes in antimicrobial, antifungal and antiviral therapy .
- Liposomes in tumour therapy.
- It is a carrier of small cytotoxic molecules.
- Vehicle for macromolecules as cytokines and genes.

Conclusion:- Liposomes are one of the unique novel drug delivery system, which have potential use in controlled&targetted drug delivery. Liposomes can be administered orally, parentally& topically and also used in cosmetic &hair technologies, sustained release formulations, diagnostic purpose. These are good carriers in gene delivery. In recent years liposomes are widely used as carriers for targetted drug delivery.

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