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LIPOSOMES: AN OVERVIEW

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ABSTRACT

Liposomes, sphere-shaped vesicles consisting of one or more phospholipid bilayers, were first described in the mid-60s. The term liposomes cover a very large number of different structures, but it can be defined as a lipid bilayer structure or a membrane that encloses an internal aqueous volume. Today, they are a very useful reproduction, reagent, and tool in various scientific disciplines, including mathematics and theoretical physics, biophysics, chemistry, colloid science, biochemistry, and biology. The site avoidance and site-specific drug targeting therapy could be achieved by formulating a liposomal product, so as to reduce the cytotoxicity of many potent therapeutic agents. Due to their size and hydrophobic and hydrophilic character, liposomes are promising systems for drug delivery. These advances have led to numerous clinical trials in such diverse areas as the delivery of anti-cancer, anti-fungal and antibiotic drugs, the delivery of gene medicines, and the delivery of anesthetics and anti-inflammatory drugs. The significant contribution of liposomes as drug delivery systems in the healthcare sector is known by many clinical products, e.g., Doxil®, Ambisome®, DepoDur™, etc. We can look forward to many more clinical products in the future.

KEYWORDS: Liposomes, phospholipids, drug encapsulation, applications.

INTRODUCTION

A liposome is a spherical vesicle having at least one lipid bilayer. These are composite structures made of phospholipids (natural or synthetic) and may contain small amounts of

other molecules. They usually have one or more concentric membranes. Liposomes can vary in size from range of 0.01 to 5.0 µm in diameter. Various therapeutic agents like anticancer drugs, vaccines, antimicrobials, genetic materials, proteins and macromolecules can be encapsulated within the bilayered vesicles. Liposomes can trap both hydrophobic and hydrophilic compounds, avoid decomposition of the entrapped combinations, and release the entrapped at designated targets.[1]

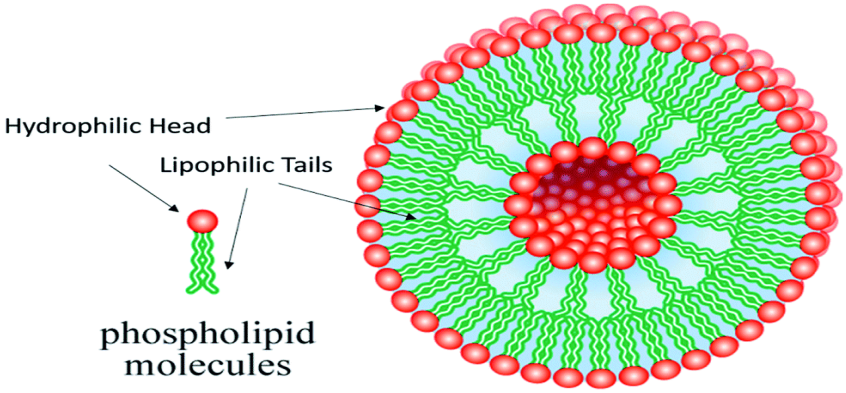

Fig. 1: Structure of liposome.[2]

A liposome design may employ surface ligands for attaching to unhealthy tissue. Targeting to specific sites (such as cells or organelles) is a key functional aspect of liposomes as drug delivery systems. This has traditionally been achieved by so-called active or passive targeting. Technical requirements for the two approaches are different: In passive targeting, liposome transport and delivery is guided by the natural distribution patterns of liposomes. PEGylated (stealth) liposomes are an example of vehicles that have been used in passive-targeting approaches. Active targeting, on the other hand, refers to the modification of the liposomes' natural distribution patterns. One way of achieving this is by attaching ligands to liposomes that recognize and bind to specific molecular and macromolecular cues on the surface of target cells. Ligands that have been used to achieve active targeting include the glycoprotein transferrin, the organic compound folic acid [3] and peptides of tryoptophan, threonine and tyrosine.

Liposomes represent versatile and advanced nanodelivery systems for a wide range of biologically active compounds. The final amount of the encapsulated drug is affected by a selection of an appropriate preparation method providing a preparation of liposomes of various size, lamellarity and physicochemical properties. The entrapment of the drugs, both hydrophilic and hydrophobic, into the liposomes is used to bypass the frequent generic toxicity associated with the drug as often seen in cancer drugs. Thus, it represents a very

effective route that enhances the drug therapeutic effect. In addition, liposomes are recently used as therapeutic agents to treat a disease because increased gene transfer efficiencies have been obtained via liposomal gene vectors in gene therapy. The benefits and limitations of liposome drug carriers critically depend and based on physicochemical and colloidal characteristics such as size, composition, loading efficiency and stability, as well as their biological interaction with the cell membranes.

➢ **Discovery**

The word "Liposome'' derives from two Greek words: Lipo ("fat") and soma ("body"); it is so named because its composition is primarily of phospholipid. Liposomes were first described by British hematologist Alec D Bangham in 1961 at Babraham Institute in Cambridge.[4] the following years, a variety of enclosed phospholipid bilayer structures were defined which were initially called bangosomes and then liposomes. Liposomes were the first nanoscale drug to be approved for clinical use in 1995. Since then, the technology has grown considerably, and pioneering recent work in liposome-based delivery systems has brought about remarkable developments with significant clinical implications. This includes long-circulating liposomes, stimuli responsive liposomes, nebulized liposomes, elastic liposomes for topical, oral and transdermal delivery and covalent lipid-drug complexes for improved drug plasma membrane crossing and targeting to specific organelles.[5]

❖ **Composition of liposomes**

A. Phospholipids

Naturally occurring phospholipids used in Liposomes 1. Phosphatidylcholine 2. Phosphatidylethanolamine 3. Phsphatidylserine

Synthetic phospholipids used in the Liposomes are 1. Disloyal phosphatidylcholine 2. Destroy phosphatidylcholine 3. Disloyal phosphatidylethanolamine

B. Cholesterol

Cholesterol can be incorporated into phospholipids membrane in very high concentration up to 1:1 or 2:1 molar ratios of cholesterol to phospatidylcholine. Being an amphipathic molecule, cholesterol inserts into the membrane with its hydroxyl group of cholesterol oriented towards the aqueous surface and aliphatic chain aligned parallel to the alkyl chains in the center of the players and also it increase the separation between choline head groups and eliminates the normal electrostatic and hydrogen bonding interaction. The phospholipids

are arranged in such a way that the hydrophilic head is exposed outside and the lipophillic tails are aliened inside. This makes the Liposomes water soluble molecules.[6,7]

- **Mechanism**

A liposome has an aqueous solution core surrounded by a hydrophobic membrane, in the form of a lipid bilayer; hydrophilic solutes dissolved in the core cannot readily pass through the bilayer. Hydrophobic chemicals associate with the bilayer.

Fig. 2: Drug release from liposome.

A liposome can be hence loaded with hydrophobic and/or hydrophilic molecules. To deliver the molecules to a site of action, the lipid bilayer can fuse with other bilayers such as the cell membrane, thus delivering liposome content. Liposomes are used as models for artificial cells. The use of liposomes for transformation or transfection of DNA into a host cell is known as lipofection.

Drug release rates have important implications for the therapeutic activities of all types of drug delivery systems, including liposomes. It is important to keep in mind that drug entrapped in liposomes is not bioavailable; it only becomes bioavailable when it is released. Hence the ability of accumulated liposomes to increase the local bioavailable drug concentrations, and increase the therapeutic outcome, only occurs when the rate of release rate of entrapped drug from the liposomes is optimized.[8,9] The drug must be delivered to the disease site and become bioavailable at a level within its therapeutic window, and at a sufficient rate, for a sufficient period, to have optimal therapeutic activity.

- **Classification of liposomes**

The liposome size can vary from very small (0.025 μm) to large (2.5 μm) vesicles. Moreover, liposomes may have one or bilayer membranes. The vesicle size is an acute parameter in determining the circulation half-life of liposomes, and both size and number of bilayers affect

the amount of drug encapsulation in the liposomes. Liposomes are classified based on their structure as[1,10]

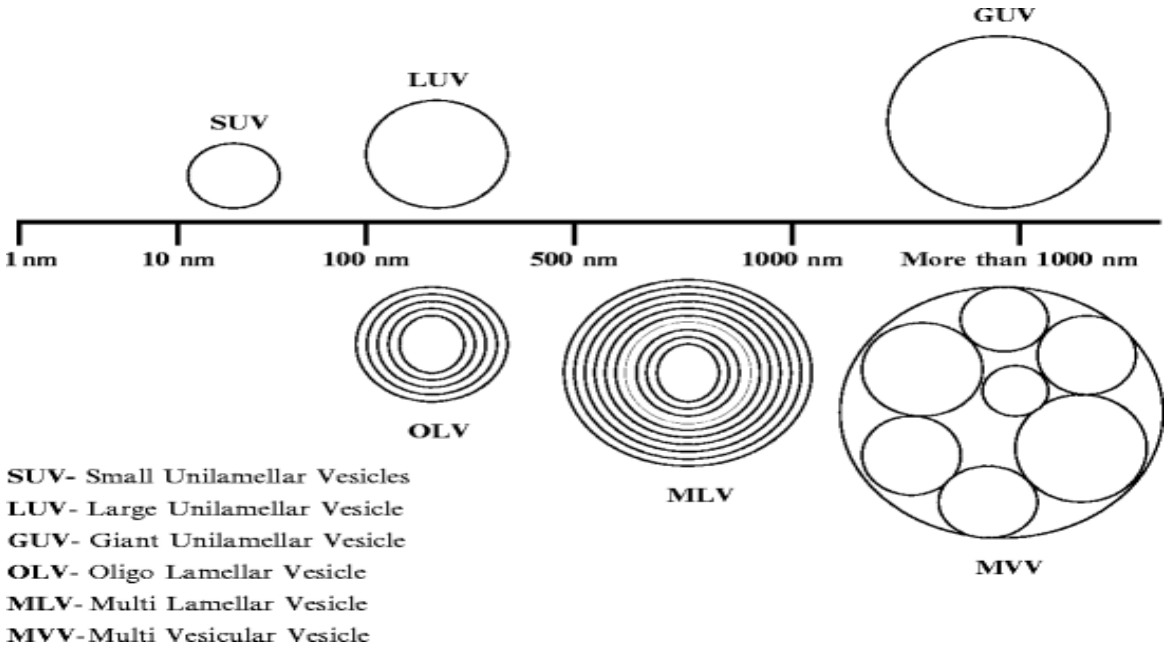

Fig. 3: Classification of liposomes.

1) Uni- Lamellar vesicles (UV)

A unilamellar liposome is a spherical chamber/vesicle, bounded by a single bilayer of an amphiphilic lipid or a mixture of such lipids, containing aqueous solution inside the chamber. Unilamellar vesicles are used to study biological systems and to mimic cell membranes.[11]

1) Small unilamellar vesicles (SUV): 20-100 nm

i. Medium unilamellar vesicles (MUV)
ii. Large unilamellar vesicles (LUV): >1µm
iii. Giant unilamellar vesicles (GUV): > 1µm

2) Multi- Lamellar Vesicles (MLV): 0.5 µm

These are the liposomes with several lamellar phase lipid bilayers. Multilamellar vesicles possess onion-like structure.

3) Oligo-Lamellar Vesicles (OLV)

Oligolamellar vesicles composed of between two and five concentric lamellae.

4) Multi- Vesicular liposomes (MVL)

Classically, several unilamellar vesicles will form on the inside of the other with smaller size, making a multilamellar structure of concentric phospholipid spheres separated by layers of water.[12]

❖ **Preparation methods**

There are a few parameters that should be considered during the method selection

1) The physicochemical characteristics of the material to be entrapped and those of the liposomal ingredients,
2) The nature of the medium in which the liposomes are dispersed,
3) The effective concentration of the encapsulated material and its potential toxicity,
4) Additional processes involved during application (delivery of the liposomes),
5) Optimum size, polydispersity and shelf-life of the liposomes for the intended application
6) Batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products.

➢ **Mechanism of vesicle formation**

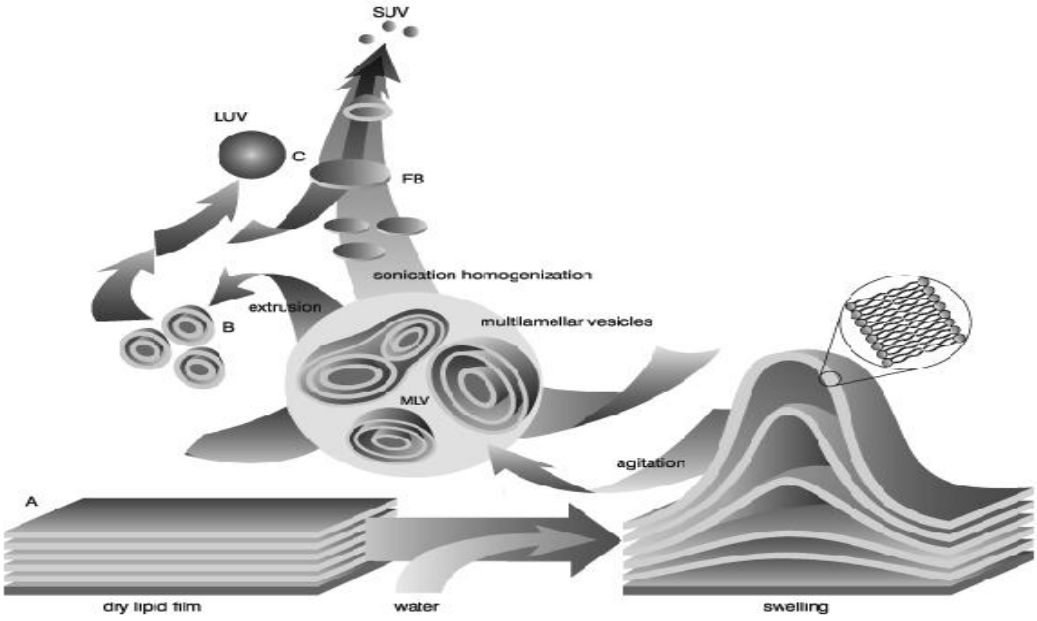

Fig. 4: Mechanism of vesicle formation.

Liposomes (lipid vesicles) are formed when thin lipid films or lipid cakes are hydrated and stacks of liquid crystalline bilayers become fluid and swell. The hydrated lipid sheets detach during agitation and self-close to form large, multilamellar vesicles (LMV) which prevents interaction of water with the hydrocarbon core of the bilayer at the edges. Once these particles have formed, reducing the size of the particle requires energy input in the form of sonic energy (sonication) or mechanical energy (extrusion).[13]

Handling of liposomes

The lipids used in the preparation of Liposomes are unsaturated and hence susceptible to oxidation. Also volatile solvents such as chloroform which is used will tend to evaporate from the content. Thus Liposomes must be stored in an inert atmosphere of nitrogen, and in the dark, in glass vessels with a securely fastened cap.

- **All the methods of preparing the liposomes involve four basic stages**[1]

1. Drying down lipids from organic solvent.
2. Dispersing the lipid in aqueous media.
3. Purifying the resultant liposome.
4. Analyzing the final product.

Method of liposome preparation and drug loading

The following methods are used for the preparation of liposome

A. Passive loading techniques
B. Active loading technique.

A. Passive loading techniques include three different methods[14,15]

1. Mechanical dispersion method-
   i. Sonication.
   ii. French pressure cell: extrusion.
   iii. Freeze-thaw sonication
   iv. Lipid film hydration by hand shaking, non-hand shaking or freeze drying.
   v. Micro-emulsification.
   vi. Membrane extrusion.
   vii. Dried reconstituted vesicles
2. Solvent dispersion method
   i. Ether injection (solvent vaporization)
   ii. Ethanol injection
   iii. Reverse phase evaporation method
   iv. Double emulsification
3. Detergent removal method (removal of nonencapsulated material)
   i. Dialysis
   ii. Column chromatography

1. Mechanical dispersion method

The following are types of mechanical dispersion methods:

Sonication

This method reduces the size of the vesicles and imparts energy to lipid suspension. Disruption of LMV suspensions using sonic energy (sonication) typically produces small, unilamellar vesicles (SUV) with diameters in the range of 15-50nm. The most common instrumentation for preparation of sonicated particles are bath and probe tip sonicators.

The probe sonicator is used for suspensions which require high energy in small volume. (eg: high concentration of lipids or viscous aqueous phase) The bath sonicator is used for large volume of dilute lipids. Cup-horn sonicators, although less widely used, have successfully produced SUV. Probe tip sonicators deliver high energy input to the lipid suspension but suffer from overheating of the lipid suspension causing degradation. Sonication tips also tend to release titanium particles into the lipid suspension which must be removed by centrifugation prior to use. For these reasons, bath sonicators are the most widely used instrumentation for preparation of SUV. Sonication of an LMV dispersion is accomplished by placing a test tube containing the suspension in a bath sonicator (or placing the tip of the sonicator in the test tube) and sonicating for 5-10 minutes above the Tc of the lipid. The lipid suspension should begin to clarify to yield a slightly hazy transparent solution. The haze is due to light scattering induced by residual large particles remaining in the suspension. These particles can be removed by centrifugation to yield a clear suspension of SUV. Mean size and distribution is influenced by composition and concentration, temperature, sonication time and power, volume, and sonicator tuning. Since it is nearly impossible to reproduce the conditions of sonication, size variation between batches produced at different times is not uncommon. Also, due to the high degree of curvature of these membranes, SUV are inherently unstable and will spontaneously fuse to form larger vesicles when stored below their phase transition temperature.[13]

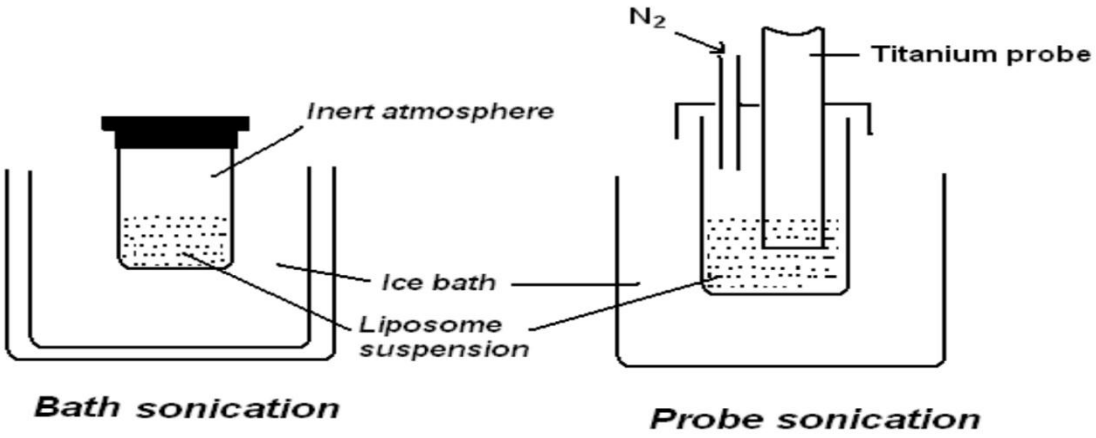

Fig. 5: Sonication apparatus.[16]

French pressure cell

French pressure cell is invented by 'Charles Stacy French'. In this technique the large vesicles are converted to small vesicles under very high pressure. This technique yields uni or oligo lamellar liposomes of intermediate size (30-80 nm in diameter depending on applied pressure). These liposomes are more stable as compared to sonicated liposomes. The French pressure cell is constructed from stainless steel and is capable of withstanding very high pressures, even up to 20,000-40,000 psi. The body of the cell contains a pressure chamber, an outlet, a piston, bottom seal, etc. both the piston and the bottom seal contain an O-ring each, which enables in tight sealing the pressure cell.[17]

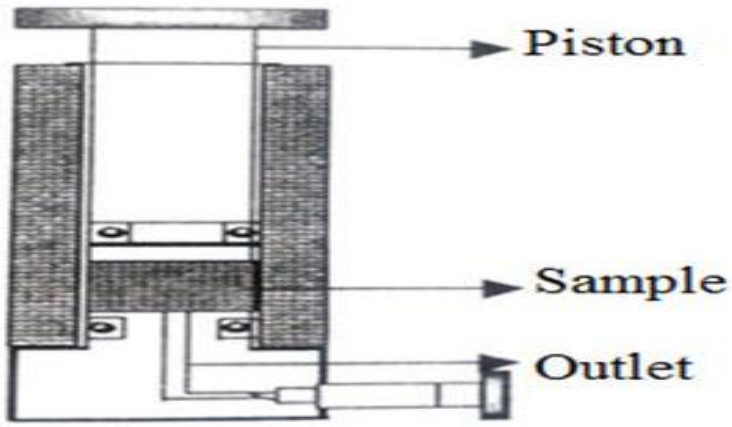

Fig. 6: French pressure cell.

Working

1) Initially the liposome suspension is added to the pressure cell and piston is pushed into the body. Then the entire cell is turned upside down that is, by an angle of 180^0.
2) The liquid sample is then filled in the entire cavity till the outlet.

3) After filling, the bottom seal is pressed down and the pressure cell is closed.
4) The cell is brought back to upright position and the pressure is developed in the cell using a hydraulic press.
5) After sufficient pressure has been developed in the pressure cell, the valve is opened very slowly and the product is allowed to exit in a drop-wise manner.

Freeze thaw sonication (FTS)

FTS method is an addition to the classical Dried Reconstituted Vesicles method. SUV's are rapidly frozen and thawed by sanding at room temperature for 15 minutes followed by subjection to a sonication for a short duration of time. The unilamellar vesicles are formed due to the fusion of SUV throughout the processes of freezing and thawing. The entrapment efficacy varies from 20% to 30%.

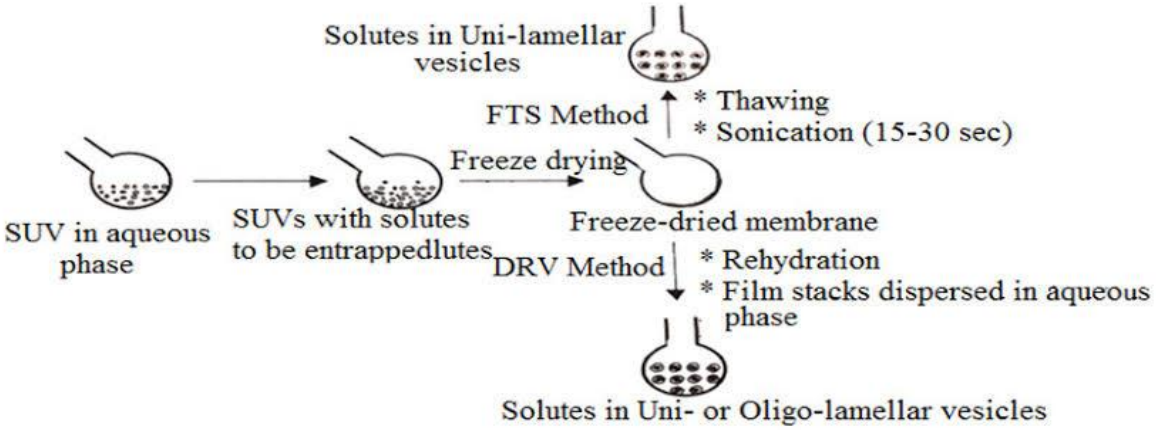

Fig. 7: FTS method.[18]

Lipid film hydration[19]

The lipids are dissolved in appropriate organic solvents. The solution is placed against a suitable surface with agitation/rotation and the organic solvent from the lipid solution is evaporated to generate a thin layer of spread- out lipids. The evaporation of organic solvent is facilitated either by increased temperature or by vacuum. Later, the dried thin lipid layer is hydrated with an aqueous phase (usually at or slightly above the transition temperature for the lipids), typically accompanied by agitation. Due to the hydrophobic nature of the tail regions of phospholipids, the liposomes are formed by the process of self- assembly as small sections of the lipids become hydrated in contact with the aqueous medium. Finally, the liposomes are purified using appropriate methods such as ultracentrifugation, gel permeation chromatography, cross-flow microfiltration, or liposome extruder purification.

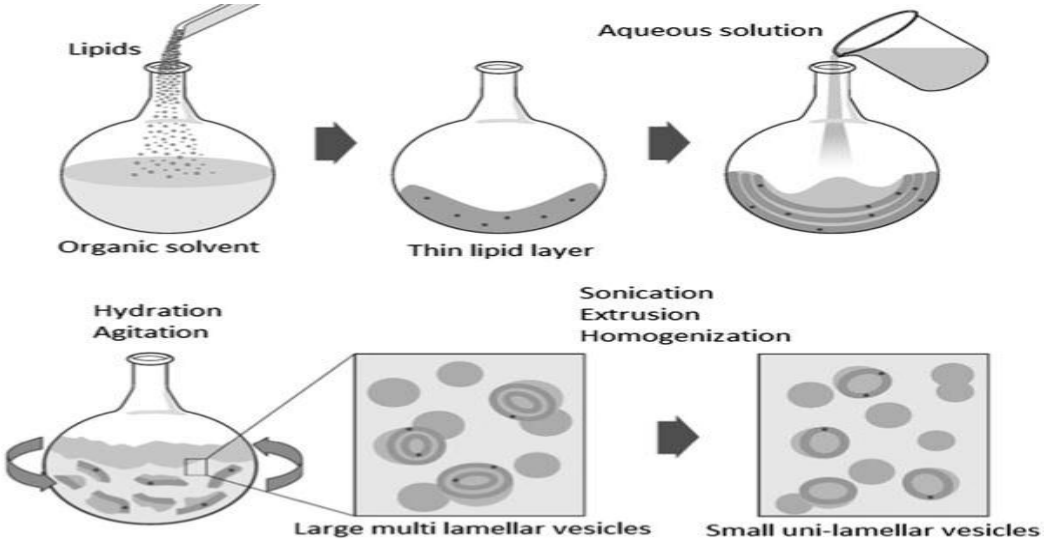

Fig. 8: **Lipid film hydration by hand shaking.**

In non- hand shaking method steam of nitrogen is used to provide agitation rather than rotationary movements. Here the lipid film is exposed to water saturated nitrogen for 15-20 minutes.

Micro- emulsification[6]

"Micro fluidizer" is used to prepare small MLVs from concentrated Lipid dispersion. Micro fluidizer pumps the fluid at very at very high pressure (10,000 psi), through a 5 micrometer orifice. Then, it is forced along defined micro channels which direct two streams of fluid collide together at the right angles at a very high velocity, thereby affecting an efficient transfer of energy. The lipids can be introduced into the fluidizer, either as large MLVs or as the slurry of a hydrated lipid in organic medium. The fluid collected can be recycled through the pump and the interaction chamber until vesicles of spherical dimensions are obtained. After a single pass, the size of vesicles is reduced to a size 0.1 and 0.2um.

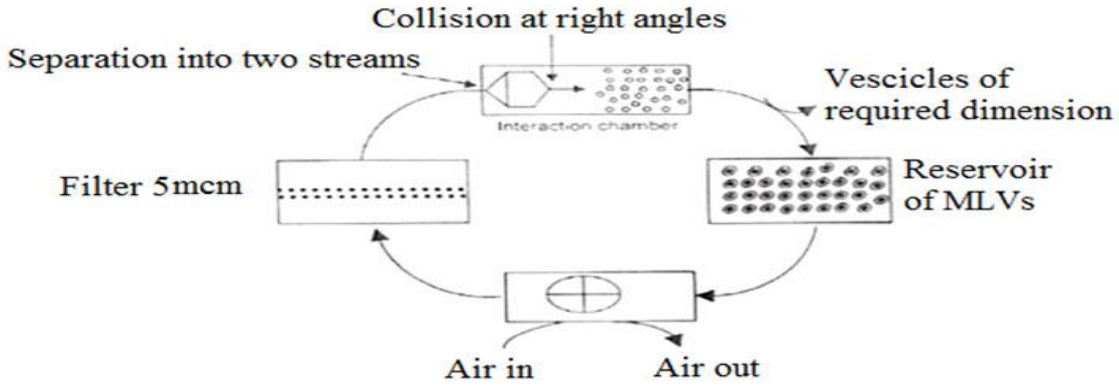

Fig. 9: Liposomes prepared by Micro- emulsification.

Membrane extrusion

Extrusion is a technique where the liposome suspension is passed through a membrane filter of defined pore size.[20] The extrusion apparatus was made up of a Waters Millipore® 510 HPLC pump (Waters, Milford, MA, USA), a guard column (Upchurch Scientific, Oak Harbour, WA, USA), a stainless steel replaceable frit (filter) with a pore size of 2 µm and polyethersulfone membrane filters of different pore sizes (0.5, 0.2 and 0.1 µm) (Sartorius AG, Goettingen, Germany). The assembly of these apparatus for extrusion process and placement of the membrane filter in the guard column are shown in Figure 10 and Figure 11, respectively.[21]

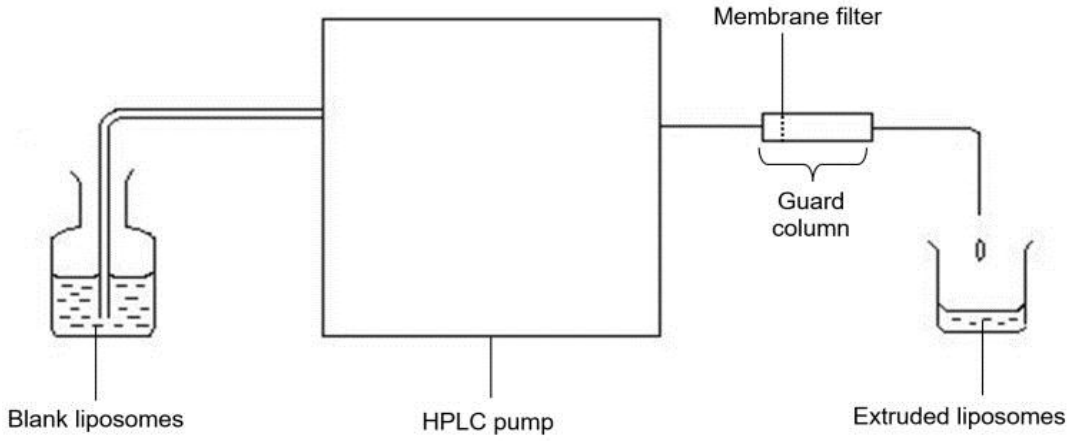

Fig. 10: Assembly of instrument used for the extrusion process.

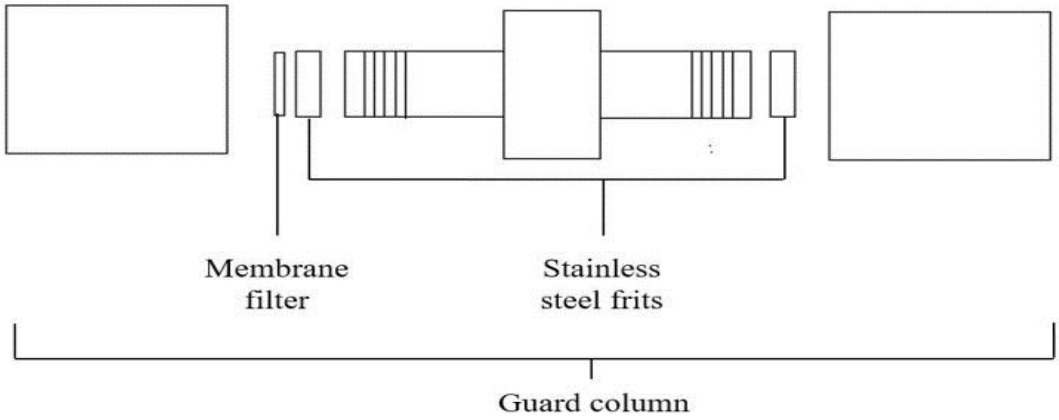

Fig. 11: Placement of membrane filter in the guard column.

A single pass through the filter converts a suspension of heterogeneous size liposomes with a substantial portion having sizes greater than 1 micron to a relatively homogeneous population having sizes less than about 0.4 micron. One or more additional passes through the filter, especially in an inside-to-outside direction, produce a convergence to a final reduced average size and polydispersity. It was found that the average size of the extended liposomes was

substantially smaller than the rated pore size of the ceramic filter. This procedure has advantages over the above homogenization and sonication methods in that a variety of membrane pore sizes is available for producing liposomes in different selected size ranges, and in addition, the size distribution of the liposomes can be made quite narrow, particularly by cycling the material through the selected-size filter several times.[22]

Dried reconstituted vesicles

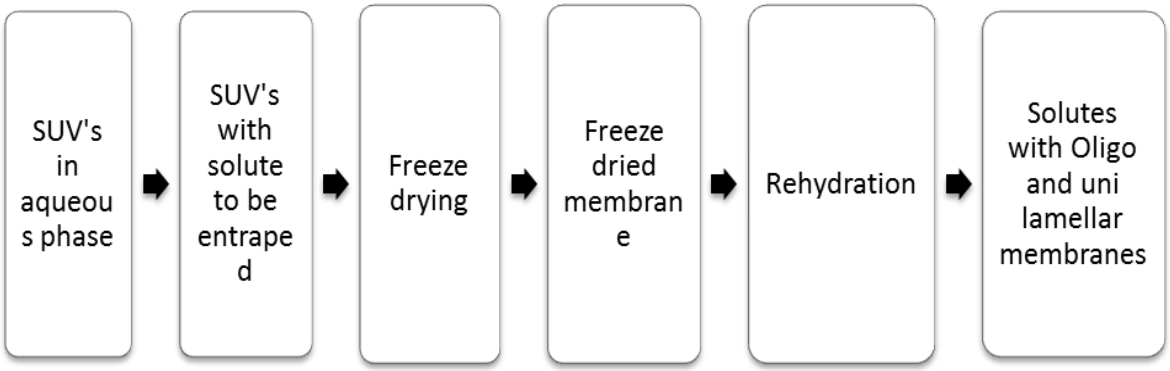

Fig. 12: Dried reconstituted vesicles.

2. Solvent dispersion method

Ether injection (solvent vaporization)- A solution of lipids dissolved in diethyl ether or ether-methanol mixture is gradually injected to an aqueous solution of the material to be encapsulated at 55°C to 65°C or under reduced pressure. The consequent removal of ether under vacuum leads to the creation of liposomes. The main disadvantages of the technique are that the population is heterogeneous (70 to 200 nm) and the exposure of compounds to be encapsulated to organic solvents at high temperature.[23,24]

Ethanol injection- A lipid solution of ethanol is rapidly injected to a huge excess of buffer. The MLVs are at once formed. The disadvantages of the method are that the population is heterogeneous (30 to 110 nm), liposomes are very dilute, the removal all ethanol is difficult because it forms into azeotrope with water, and the probability of the various biologically active macromolecules to inactivate in the presence of even low amounts of ethanol is high.[25]

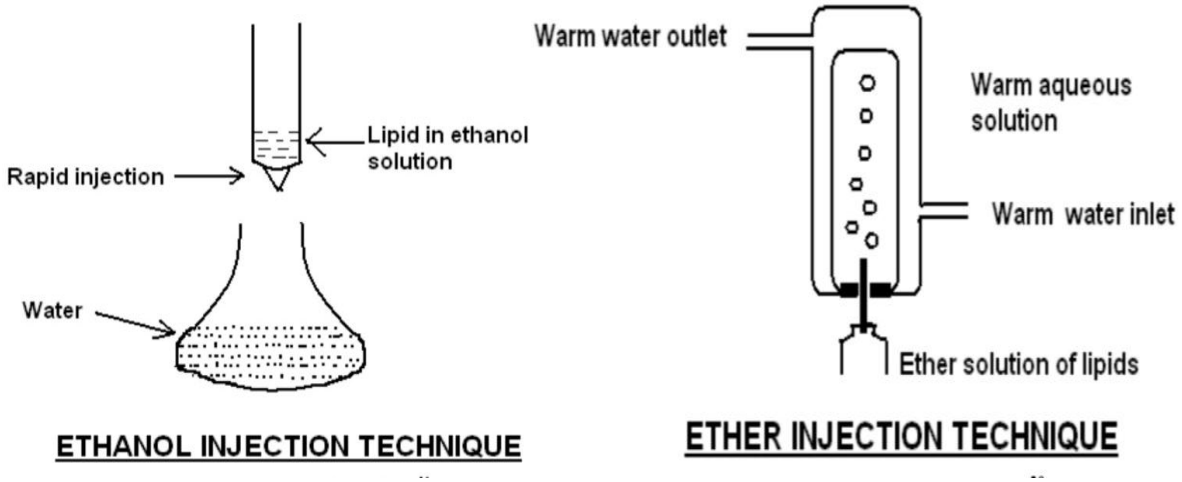

Fig. 13: Ethanol injection technique and Ether injection technique.[16]

Reverse phase evaporation method

This method provided a progress in liposome technology, since it allowed for the first time the preparation of liposomes with a high aqueous space-to-lipid ratio and a capability to entrap a large percentage of the aqueous material presented. Reverse-phase evaporation is based on the creation of inverted micelles. These inverted micelles are shaped upon sonication of a mixture of a buffered aqueous phase, which contains the water-soluble molecules to be encapsulated into the liposomes and an organic phase in which the amphiphilic molecules are solubilized. The slow elimination of the organic solvent leads to the conversion of these inverted micelles into viscous state and gel form. At a critical point in this process, the gel state collapses, and some of the inverted micelles were disturbed. The excess of phospholipids in the environment donates to the formation of a complete bilayer around the residual micelles, which results in the creation of liposomes. Liposomes made by reverse phase evaporation method can be made from numerous lipid formulations and have aqueous volume-to-lipid ratios that are four times higher than hand-shaken liposomes or multilamellar liposomes.[1]

Double emulsification

In this process, an active ingredient is first dissolved in an aqueous phase (W1) which is then emulsified in an organic solvent of a polymer to make a primary W1/O emulsion. This primary emulsion further mixed in an emulsifier- containing aqueous solution (W2) to make a W1/W2 double emulsion. The removal of the solvent leaves microspheres in the aqueous continuous phase, making it possible to collect them by filtering or centrifuging.

3. Detergent removal method

The micellar dispersion is then subjected to one of the following methods- i) Dialysis ii) Column chromatography.

Dialysis method

Dialysis is the simplest procedure used for the removal of the unbound drug, except when macromolecular compounds are involved. Dialysis technique require no complicated or expensive equipment. Dialysis is effective in removing nearly all of the free drug with a sufficient number of changes of the dialyzing medium.[26]

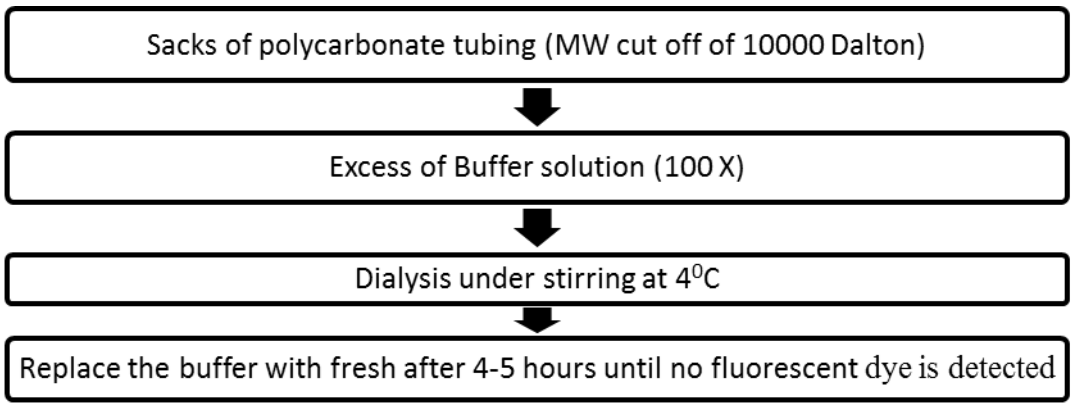

Fig. 14: Dialysis method.

Column chromatography

Phospholipid in the form of either sonicated vesicles or as a dry film, at a molar ratio of 2:1 with deoxycholate form unilamellar vesicles of 100nm on removal of deoxycholate by column chromatography. In column chromatographic separation technique, Sephadex G-50 is most widely used material.

C. Active loading technique[26]

Two steps process generates this pH imbalance and active (remote) loading.

1. Vesicles are prepared in low pH solution, thus generating low pH within the liposomal interiors
2. Followed by addition of the base to extraliposomal medium.

Basic compounds, carrying amino groups are relatively lipophilic at high pH and hydrophilic at low pH.

a) After drying in process
b) Film/cake of lipid is formed

c) Stacks of lipid bilayer formed
d) Swelling in fluid
e) Sheet is self-close
f) Loading of drug on pH- Gradient technique
g) Formation of bilayer (liposomes)

1. Lyophilization

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

2. Pro-liposomes

To increase the surface area of dried lipid film and to facilitate continuous hydration and lipid is dried over the finally divided particulate support that is NaCl, sorbitol, or other polysaccharides. These dried lipid coated particulates are called as proliposomes. Proliposomes form dispersion of MLVs on addition of water, where support is rapidly dissolved and lipid film hydrate to form MLVs.

- **Production of liposomes in industries**[27]

Most of the methods proposed for the preparations of liposomes are not suitable for the for scale up purpose as the complications like the nature of solvent, reproducibility, sterility plays a major role in ruling out many of the above proposed methods.

Most commonly used methods at industrial level are

1. Detergent dialysis method:
2. Microfludisation method.
3. Proliposomes
4. Freeze drying method

- **Advantages**[28,29,30]

1. Provides selective passive targeting to tumor tissues
2. Increased efficacy and therapeutic index
3. Increased stability via encapsulation

4. Liposomes are non-toxic, flexible, biocompatible, biodegradable and non-immunogenic for systemic and non-systemic administrations
5. They reduce the toxicity of encapsulated agent.
6. They help to reduce the exposure of sensitive tissues to toxic drugs.
7. Used as a carrier for controlled and sustained drug delivery
8. Can be made into various sizes
9. Ability to carry large drug payloads
10. They can incorporate both hydrophobic and hydrophilic drugs in them.

➢ **Disadvantages**[31]

1. Low solubility
2. Short half life
3. Production cost is high
4. Sometimes phospholipids undergoes oxidation and hydrolysis
5. Leakage and fusion of encapsulated drug/molecules
6. Batch to batch variation

Applications[32]

- Cancer chemotherapy
- Gene therapy
- Ophthalmic delivery of drugs
- Liposomes as carriers for vaccines
- Liposomes as carrier of drug in oral treatment
- Liposomes for topical applications
- Liposomes for pulmonary delivery
- Against Leishmaniasis
- Lysosomal storage disease
- Metal storage disease
- Cell biological application

CONCLUSION

Today, liposomes are a very useful reproduction, reagent, and tool in various scientific disciplines, including mathematics and theoretical physics, biophysics, chemistry, colloid science, biochemistry, and biology. These closed bilayer phospholipid vesicles have

witnessed many technical advances in recent years since their first development in 1965. The ability of liposomes to encapsulate hydrophilic or lipophilic drugs has allowed these vesicles to become useful drug delivery systems. In clinical applications, liposomal drugs have been proven to be most useful for their ability to "passively" accumulate at sites of increased vasculature permeability, when their average diameter is in the ultrafilterable range (b200 nm in diameter), and for their ability to reduce the side effects of the encapsulated drugs relative to free drugs. This has resulted in an overall increase in therapeutic index, which measures efficacy over toxicity. The role of liposomes as a drug delivery system is to provide drugs in a controlled manner, reduce toxicity, and increase the efficacy of encapsulated drugs. Based on the pharmaceutical applications and available products, we can say that liposomes have definitely established their position in modern delivery systems. A number of liposomes (lipidic nanoparticles) are on the market, and many more are in the pipeline.

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