

LIPOSOME: A POWERFUL APPROACH FOR VESICULAR DRUG DELIVERY SYSTEM

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ABSTRACT

The first closed bilayer phospholipid system called Liposomes, was described in 1964 and soon was proposed as drug delivery system. Liposome was found by Alec Bangham of Babraham Institute in Cambridge, England in 1965. In 1965s, it was well recognized that microscopic lipid vesicles, known as liposomes, could be utilized to encapsulate drugs and dyes for the purpose of systemic administration and drug targeting. Liposomes have been widely investigated since 1970 as drug carriers for improving the delivery of therapeutic agents to specific sites in the body. In 1990; drugs with liposome and Amphotericin B were approved by Ireland. In 1995 America F.D.A approved liposor doxodubicin. The liposome a microscopic spherical

particle formed by a lipid bilayer enclosing an aqueous compartment. An artificial microscopic vesicle consisting of an aqueous core enclosed in one or more phospholipid layers, used to convey vaccines, drugs, enzymes, or other substances to target cells or organs. This review discusses the mechanism of liposome formation, structural components of liposome, classification, preparation method, pharmacokinetics, targeting, advantages, disadvantages, limitation, factors affecting the formation, characterization, potential applications of liposomes in drug delivery with examples of formulations approved for clinical use and products in clinical trials.

KEYWORDS: lipophilic, phospholipids are microscopic vesicles, clinical trial, targeting.

INTRODUCTION

Liposomes are composed of small vesicles of phospholipids encapsulating an aqueous space ranging from about 0.03 to 10 μm in diameter. The membrane of liposome is made of

phospholipids, which have phosphoric acid sides to form the liposome bilayers.^[1] Liposomes were first described by British haematologist Dr Alec D Bangham in 1961 (published 1964), at the Babraham Institute, in Cambridge. They were discovered when Bangham and R. W. Horne were testing the institute's new electron microscope by adding negative stain to dry phospholipids (Kamble R and Pokharkar V. B et al. 2010, Bangham A. D, 1983). The name liposome is derived from two Greek words: 'Lipos' meaning fat and 'Soma' meaning body.^[2] A liposome can be formed at a variety of sizes as unilamellar or multi-lamellar construction, and its name relates to its structural building blocks, phospholipids, and not to its size.^[3] Liposome was discovered about 40 years ago by Bangham and coworkers and was defined as microscopic spherical vesicles that form when phospholipids are hydrated or exposed to an aqueous environment. Liposomes are microscopic vesicles composed of a bilayer of phospholipids or any similar amphipathic lipids.^[4] They can encapsulate and effectively deliver both hydrophilic and lipophilic substances and may be used as a non-toxic vehicle for insoluble drugs. Liposomes can be manufactured in different lipid compositions or by different methods show variation in parameters. Size, size distribution, surface electrical potential, number of lamella, encapsulation efficacy, surface modification showed great advantage to produce liposomes of different mechanisms, kinetic properties and biodistribution.^[5,6]

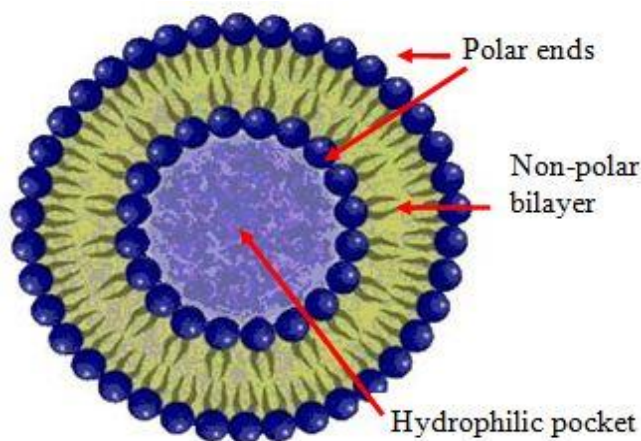


Fig 1. Structure of liposomes.^[4]

Advantages of Liposome

1. Liposome is increased efficacy and therapeutic index of drug (Actinomycin D).
2. Liposome is increased stability via encapsulation. Liposomes are biocompatible, completely biodegradable, non-toxic, flexible and nonimmunogenic for systemic and non-systemic administrations.
3. Liposome are reduction in toxicity of the encapsulated agent (Amphotericin B, Taxol).

4. Liposomes help to reduce exposure of sensitive tissues to toxic drugs.
5. Biocompatible, completely biodegradable, non-toxic, flexible and nonimmunogenic.
6. Alter the pharmacokinetic and pharmacodynamic property of drugs (reduced elimination, increased circulation life time).
7. Provides selective passive targeting to tumour tissue (liposomal doxorubicin).^[8,9, 10]

Disadvantage^[9, 10]

1. Production cost is high.
2. Leakage and fusion of encapsulated drug / molecules.
3. Sometimes phospholipid undergoes oxidation and hydrolysis like reaction.
4. Short half-life.
5. Low solubility.
6. Less stable. The development of liposomes at industrial level is difficult due to its physiological and physicochemical instability.
7. They are prone to degradation by oxidation and hydrolysis.
8. Production cost is high.
9. Leakage and fusion of encapsulated drug / molecules.
10. Low solubility
11. Fewer stable.

Composition of Liposomes

A. Phospholipids

Phospholipids are the major structural component of biological membranes, where two type of phospholipids exist-phosphodiglycerides and sphingolipids. The most common phospholipid is phosphatidylcholine (pc) molecule. Molecule of phosphatidylcholine are not soluble in water and in aqueous media they align themselves closely in planar bilayer sheets in order to minimize the unfavorable action between the bulk aqueous phase and long hydrocarbon fatty chain.^[11,12] The glycerol containing phospholipids are most common used component of liposome formulation and represent greater than 50% of weight of lipid in biological membranes. These are derived from phosphatidic acid.^[13]

Examples of naturally occurring phospholipids used in liposomes are^[12, 13]

1. Phosphatidyl choline (Lecithin) (PC).
2. Phosphatidyl ethanolamine (Cephalin) (PE)
3. Phosphatidyl serine (PS).

4. Phosphatidyl inositol (PI)
5. Phosphatidyl Glycerol (PG)

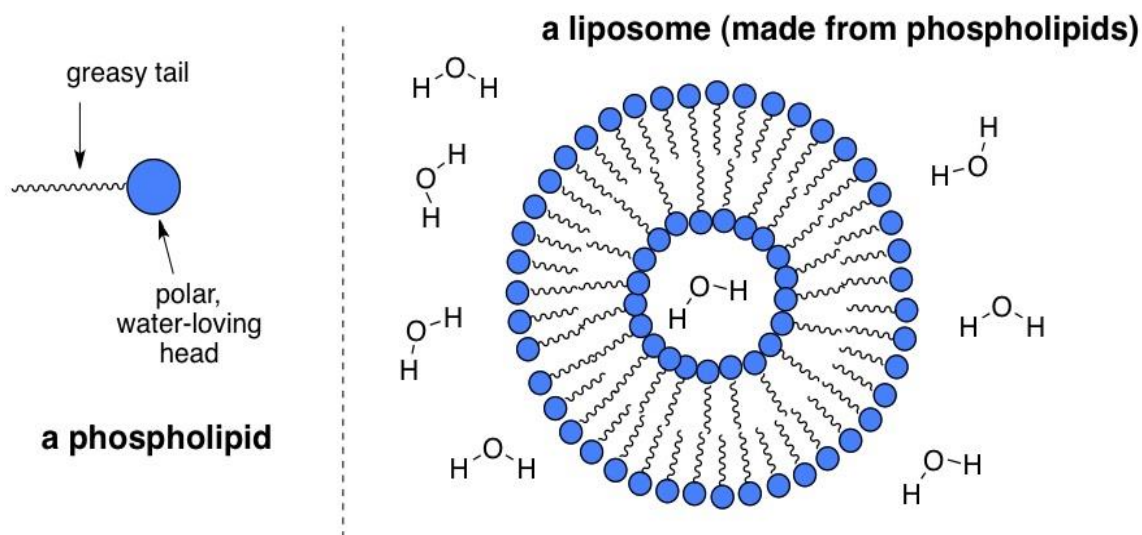


Fig 2. Structure of liposomes made from phospholipids.

Examples of Synthetic phospholipids used in the liposomes are

1. Dioleoyl phosphatidylcholine (DOPC)
2. Distearoyl phosphatidylcholine (DSPC)
3. Dioleoyl phosphatidylethanolamine (DOPE)
4. Distearoyl phosphatidylethanolamine (DSPE).

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 phosphatidylcholine. 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 acyl chains in the center of the bilayers and also it increase the separation between choline head groups and eliminates the normal electrostatic and hydrogen bonding interaction.^[14, 15]

Mechanism of Liposome Formation

1. As liposomes are made up of phospholipids, they are amphipathic in nature and have ability to binds both aqueous and polar moiety. They have polar head and non polar tail.
2. The polar end is mainly phosphoric acid and it will bound to water soluble molecule.
3. In aqueous medium the molecules in self-assembled structure are oriented in such way that the polar portion of the molecule remain in contact with in polar environment and at

same time shields the non polar part. Liposomes are formed when the thin films are hydrated and stacks of liquid crystalline bilayers become fluid and swells.

4. Once these vesicle get formed, a change in vesicle shape and morphology required energy input in the form of Sonic energy to get SUVs and mechanical energy to get LUVs.
5. However, in aqueous mixtures these molecules are able to form various phases, some of them are stable and other remains in metastable form.^[16,17]

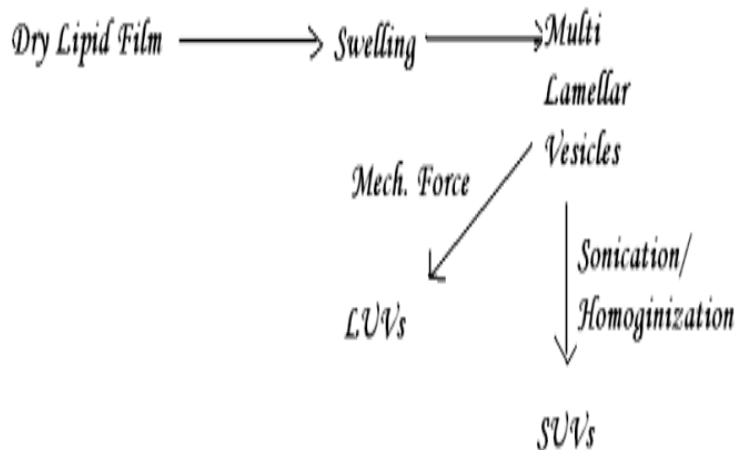


Fig3. Mechanism of liposome preparation.^[18]

Formulation of Liposomes

Liposomes are made from pure lipids or a combination of lipids. The lipids commonly employed in liposome formulations are phospholipids. Liposomes have been prepared from a variety of synthetic and naturally occurring phospholipids, they generally contain cholesterol. The incorporation of cholesterol into the lipid bilayer membrane generally enhances the stability of liposomes in serum, reduces the permeability of the membranes to water soluble molecules and increases the fluidity or microviscosity of the bilayer.^{18,19} The most commonly used phospholipids in liposome preparation are egg phosphatidylcholine, synthetic dipalmitoyl-DL- α -phosphatidylcholine, brain and synthetic phosphatidylserine, sphingomyelin, phosphatidylinositol and ovolcithin. Usually, a zwitterionic or non-ionic lipid is used as the basic lipid for the preparation of liposomes. The net surface charge of liposome can be modified by the incorporation of positively charged lipids such as stearylamine, or negatively charged lipids such as diacetylphosphate, phosphatidyl glycerol or phosphatidyl serine. The presence of negatively or positively charged lipids lead to a greater overall volume for aqueous entrapment and reduces the likelihood of aggregation after preparation of the liposomes.^[20,21]

Technology of Liposome Production

Since the early 1970s many hundreds of drugs, including antitumour and antimicrobial agents, chelating agents, peptide hormones, enzymes, other proteins, vaccines and genetic materials, have been incorporated into the aqueous or lipid phases of liposomes of various sizes, compositions and other characteristics by an ever-increasing number of techniques.^[20,21] Liposomes have evolved from mere experimental tools of research to industrially manufactured products for clinical and veterinary use. This success depends on advanced techniques to obtain efficient drug entrapment and increased stability of the products.^[22, 23]

Conventional Method

The Convectional method was first described in detail by Bangham et al for the preparation of MLVs. In the procedure; the phospholipids are dissolved in an organic solvent (usually a chloroform/methanol mixture) and deposited from the solvents as a thin film on the wall of a round bottom flask by use of rotary evaporation under reduced pressure. MLVs form spontaneously when an excess volume of aqueous buffer containing the drug is added to the dried lipid film.^[22,23] Drug containing liposomes can be separated from nonsequestered drug by centrifugation of the liposomes or by gel filtration. The time allowed for hydration of the dried film and conditions of agitation are critical in determining the amount of the aqueous buffer (or drug solution) that will be entrapped within the internal compartments of the MLVs. For instance, it is reported that more of the aqueous phase can be sequestered when the lipid is hydrated for 20 hours with gentle shaking, compared with a hydration period of two hours, with vigorous shaking of the flask, even though size distribution of the MLVs was unaffected. This means that slow hydration is associated with greater entrapment of aqueous volume.^[24,25]

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 are used will tend to evaporate from the container. Thus liposomes must be stored in an inert atmosphere of nitrogen, and in the dark, in glass vessels with a securely fastened cap.^[26,27]

Mechanism of Liposome Formation

Phospholipids are amphipathic having affinity for both aqueous and polar moieties molecules as they have a hydrophobic tail and a hydrophilic or polar head. The hydrophobic tail is

composed of two fatty acid chain containing 10-24 carbon atom and 0-6 double bonds in each chain. The macroscopic structures most often formed include lamellar, hexagonal or cubic phases dispersed as colloidal nanoconstructs (artificial membranes) referred to as liposomes, hexosomes or cubosomes. The most common natural polar phospholipids are phosphatidylcholine. These are amphipathic molecules in which a glycerol bridge links to a pair of hydrophobic acyl hydrocarbon chains with a hydrophilic polar head group, phosphocholine. The amphipathic nature of phospholipids and their analogues render them the ability to form closed concentric bilayers in presence of water. Liposomes are formed when thin lipid films or lipid cakes are hydrated and stacks of lipid crystalline bilayers become fluid and swell. The hydrated lipid sheets detach during agitation and self close to form large, multilamellar vesicles prevent interaction of water with the hydrocarbon core of the bilayer at the edges (Cullis P. R and Mayer L. D et al. 1989).^[28, 29]

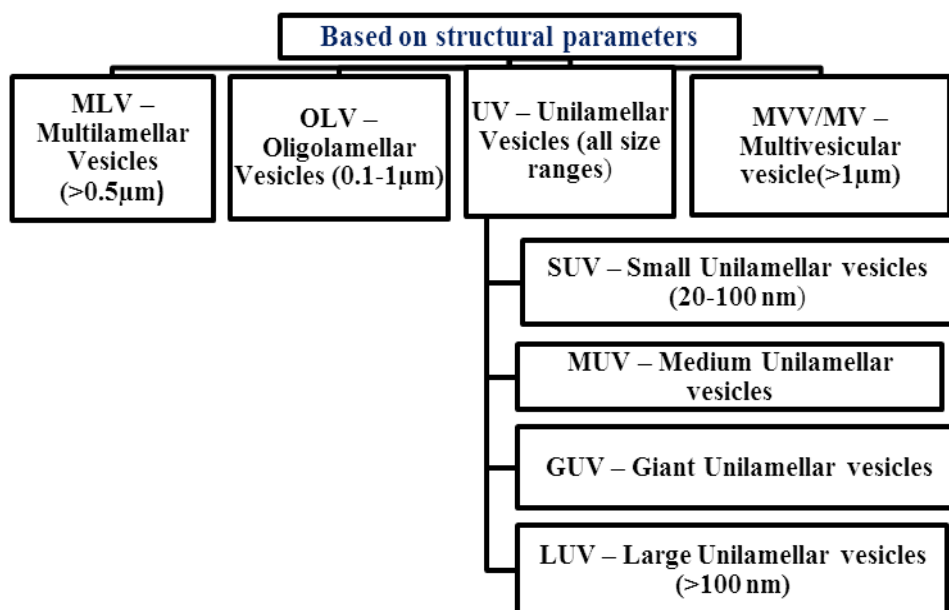


Fig4. Classification of Liposomes Base on Structural Parameters.

Types of Liposomes

1. Conventional Liposomes

These can be defined as liposomes that are typically composed of only phospholipids (neutral and/or negatively charged) and/or cholesterol. Most early work on liposomes as a drug-carrier system employed this type of liposomes.^[32,33] Conventional liposomes are a family of vesicular structures based on lipid bilayers surrounding aqueous compartments. They can vary widely in their physicochemical properties such as size, lipid composition, surface charge and number and fluidity of the phospholipid bilayers. Although manipulation of these properties is a valuable tool to modify, to a certain extent, the in vivo behavior of

conventional liposomes (i.e. stability, clearance and distribution), some in vivo behavioral features are very consistent among different conventional- liposome formulations.^[34, 35] Conventional liposomes are characterized by a relatively short blood circulation time. When administered in vivo by a variety of parenteral routes (often by intravenous administration), they show a strong tendency to accumulate rapidly in the phagocytic cells of the mononuclear phagocyte system (MPS), also often referred to as the reticuloendothelial system (RES).^[36, 37] The major organs of accumulation are the liver and the spleen, both in terms of total uptake and uptake per gram of tissue. Another interesting application of macrophage targeting involves the delivery of immunomodulators to increase the capacity of macrophages to kill neoplastic cells (Daemen T et al. 1995, Killion, J.J. and Fidler, I.J. 1994) and to increase resistance against infectious microorganisms (Ten Hagen and T.L.M et al. 1985). Conventional liposomes have also been used for antigen delivery.^[38, 39] Liposome-based vaccines have proved effective in experimental models against viral, bacterial and parasitic infections (Gregoriadis G. 1994 and Alving C.R. 1995, Kersten G.F. and Crommelin D.J.A. 1995), as well as against tumors (Bergers J.J. and Storm G 1993). Several liposomal vaccines have been tested in humans, and one of these, a liposomal hepatitis-A vaccine, has received marketing approval in Switzerland (Gluck R 1995, Gluck R et al.1992).^[39, 40, 41, 42]

2. Fusogenic Liposomes for Intracellular Delivery

Fusogenic liposomes can potentially facilitate the intracellular delivery of encapsulated drugs by fusing with the target cell. A variety of approaches can be envisioned for constructing fusogenic liposomes. Examples include the inclusion of lipids that are able to form non-bilayer phases, such as DOPE, which can promote destabilization of the bilayer, inducing fusion events. Furthermore, alterations in the lipid composition can render liposomes pH sensitive, leading to enhanced fusogenic tendencies in low pH compartments such as endosomes. Nonphospholipid fusogenic liposomes composed primarily of dioxyethylene acyl ethers and cholesterol have been shown to fuse with plasma membranes of erythrocytes and fibroblasts.^[43,44] Alternatively, efficient fusogenic liposomes can be achieved by incorporating fusogenic proteins into the liposome membrane or entrapped within liposomes. The feasibility of this approach has been demonstrated for the delivery of the diphtheria toxin A subunit using liposomes produced from influenza virus envelopes. Fusogenic peptides can be conjugated to the liposomes and may also promote intracellular delivery. The encapsulation of a 30-amino acid fusogenic peptide has recently been shown to promote relatively efficient endosomal release of propidium iodide with 20-25% of the encapsulated

propidium iodide gaining access to KB cell chromosomal DNA after 48 h. A number of studies illustrate that the above approaches to enhance the fusogenic activity of liposomes can be applied to enhance the efficiency of lipid-based gene delivery systems. For example, the addition of replication deficient adenovirus, which enhance endosomal escape, to cationic liposome plasmid DNA complexes results in an approximately fivefold increase in chloramphenicol acyl transferase activity detected in FAO hepatoma and 3T3-F442A adipocyte cells in vitro and up to a 1000-fold increase in luciferase expression in human smooth muscle cells in vitro. Similarly, the incorporation of the fusogenic protein from Sendai virus, by fusing Sendai virus with preformed DNA-containing liposomes, results in a liposome with improved gene delivery properties (Leo RJ, Huang L. 1996, Arcadio Chonna, Pieter R 1998).^[45,46]

PH Sensitive Liposomes

PH-sensitive liposomes have been developed to mediate the introduction of highly hydrophilic molecules or macromolecules into the cytoplasm. These liposomes destabilize under acidic conditions found in the endocytotic pathway, and usually contain phosphatidylethanolamine (PE) and titratable stabilizing amphiphiles. Encapsulated compounds are thought to be transported into the cytoplasm through destabilization of or fusion with the endosome membrane.^[47, 48,49]

Cationic Liposome

Cationic liposomes represent the youngest member of the liposome family. They are front-line runners among the delivery systems under development for improving the delivery of genetic material. Their cationic lipid components interact with, and neutralize, the negatively-charged DNA, thereby condensing the DNA into a more compact structure. The resulting lipid-DNA complexes, rather than DNA encapsulated within liposomes, provide protection and promote cellular internalization and expression of the condensed plasmid.^[50, 52]

Immuno-Liposomes

Have specific antibodies or antibody fragments (like Fab9 or single chain-antibodies) on their surface to enhance target site binding. Although immunoliposome systems have been investigated for various therapeutic applications, the primary focus has been the targeted delivery of anticancer agents (Storm G. et al. 1995).^{50,52} As for any particle in the bloodstream, it is difficult for immunoliposomes to leave the blood compartment at sites other than the liver and the spleen. Therefore, to guarantee accessibility of the target

receptors, local administration in body cavities has received some interest. Successful attempts have been made to prolong the half-life of immunoliposomes after intravenous administration by coating with PEG, thus giving them a greater chance to reach target sites other than MPS macrophages.^[52,53]

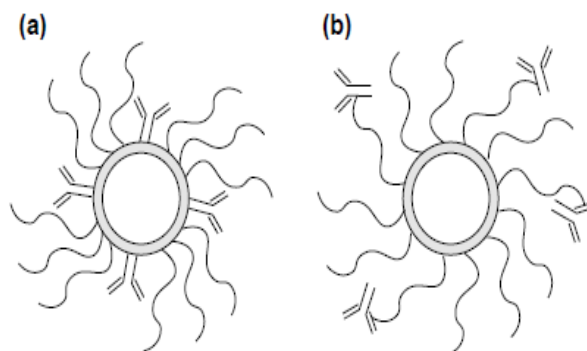


Fig5. Immobilization of antibody on PEG-liposomes by (a) direct coupling to the liposome surface (which may provide steric hindrance to antigen binding) and (b) coupling to the terminal end of PEG chains (which does not give a steric hindrance problem).^[52,53]

Method of Preparation Based on Method of Dispersion

1. Passive Loading

(A) Physical Dispersion or Mechanical Dispersion Method

Aqueous volume enclosed using this method usually 5-10%, which is very small proportion of total volume used for swelling. Therefore large quantity of water-soluble compound are wasted during swelling. On the other hand lipid soluble compound can be encapsulated to 100% efficacy, provided they are not present in quantities that are greater than the structural component of the membrane.

Lipid Film Hydration by Hand Shaking and Non Hand Shaking

Hand Shaking Method

Step1

Lipid mixture of different phospholipid and charge components in chloroform:methanol solvent mixture (2:1v/v) is prepared first and then introduced into a round bottom flask with a ground glass neck. This flask is then attached to a rotary evaporator and rotated at 60 rpm. The organic solvent are evaporated at about 30 degree Celsius or above the transition temperature of the lipids used. The evaporator is isolated from the vacuum source by closing the tap. The nitrogen is introduced into the evaporator and the pressure at the cylinder head is gradually

raised till there is no difference between inside and outside the flask. The flask is then removed from the evaporator and fixed on to the manifold of lyophilizer to remove residual solvents.^[54,55]

Hydration of Lipid Layer

After releasing the vacuum and removal from the lyophilizer, the flask is flushed with nitrogen, 5 ml of saline phosphate buffer (containing solute to be entrapped) is added. The flask is attached to the evaporator again (flushed with N₂) and rotated at room temperature and pressure at the same speed or below 60 rpm. The flask is left rotating for 30 minutes or until all lipid has been removed from the wall of the flask and has given homogenous milky-white suspension free of visible particles. The suspension is allowed to stand for a further 2 hours at room temperature or at a temperature above the transition temperature of the lipid in order to complete the swelling process to give MLVs.^[54,55,56]

Non-Shaking Vesicles

Method described by Reeves and Dowben in 1996 by which large unilamellar vesicles (LUVs) can be formed with higher entrapment volume. The procedure differs from hand shaken method in that it uses a stream of nitrogen to provide agitation rather than the rotary movements. Solution of lipid in chloroform:methanol mixture is spread over the flat bottom conical flask.^[55,56] 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 (15-20 mins). After hydration, lipid is swelled by addition of bulk fluid. The flask is inclined to one side, 10-20 ml of 0.2% sucrose in distilled water (degassed) is introduced down the side of the flask and the flask is slowly returned to upright orientation. The fluid 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 degrees Celsius. Take care not to disturb the flask in any way. After swelling the vesicles are harvested by swirling the contents, to yield a milky suspension.^[57]

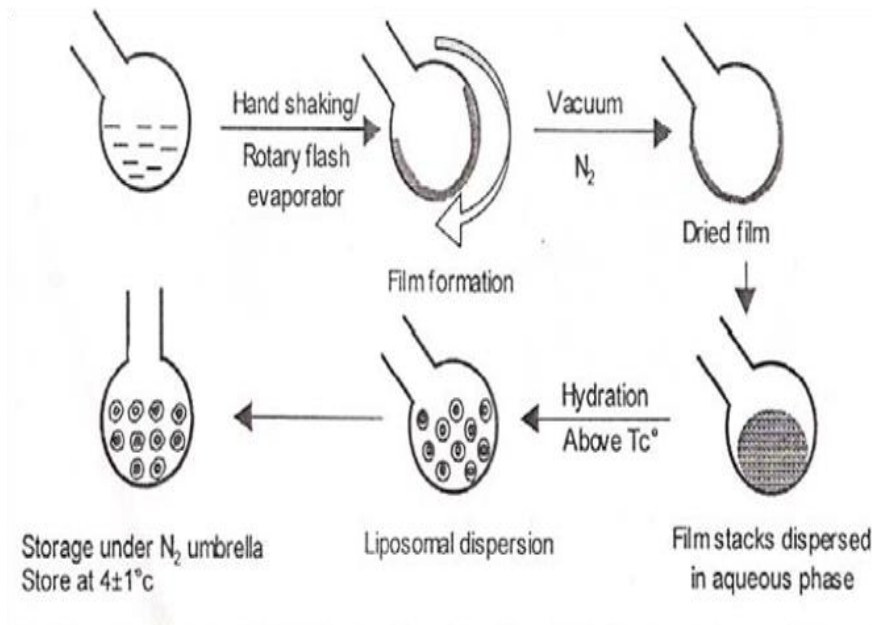


Fig 6. Method of preparation of liposomes by Hand shaking method.

Sonication

This is the method in which Multi lamellar vesicles are transformed to the small uni lamellar vesicles. The ultra sonic irradiation is provided to the MLVs to get the SUVs. There are two methods are used. a) Probe sonication method., b) Bath sonication method. The probe is employed for dispersion, which requires high energy in small volume (e.g. high conc. of lipids or a viscous aqueous phase) while is more suitable for large volumes of diluted liquid. Probe tip sonicator provides high energy input to the liquid dispersion but suffer from overheating of liposomal dispersion causing lipid degradation. sonication tip also release titanium into the liposome dispersion which will be removed from the it by centrifugation prior to use. Due to above reason most widely the bath sonicators are used. Sonication of MLVs is accomplished by placing dispersion into the the bath sonicator or placing tip fo probe sonicator into the test tube of dispersion. (5-10 min.) After sonication applied the resultant dispersion is centrifuged and according to diagram the SUVs will stay on the top and the small MLVs and aggregated lipids will get settled down. The top layer constitutes pure dispersion of SUVs with varying diameter as size is influenced by composition and concentration, temperature, sonication, volume and sonication tuning. ^[58,59]

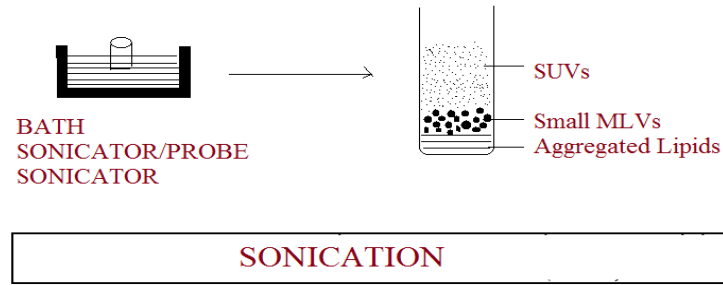


Fig 7. Method of preparation of liposomes by sonication.

a) Micro emulsification Liposomes

'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 to collide together at the right angles at a very high velocity, there by affecting an efficient transfer of energy. The lipids can be introduced into the fluidizer, either as large MLVs or as the slurry of un hydrated lipid in organic medium. The fluid collected can be recycled through the the pump and interaction chamber until vesicles of spherical dimensions are obtained. diameter After a single pass, the size of vesicles is reduced to a size 0.1 and 0.2 μm .^[58,59]

Freeze Thaw Sonication

This method is based upon freezing of a unilamellar dispersion (SUV).

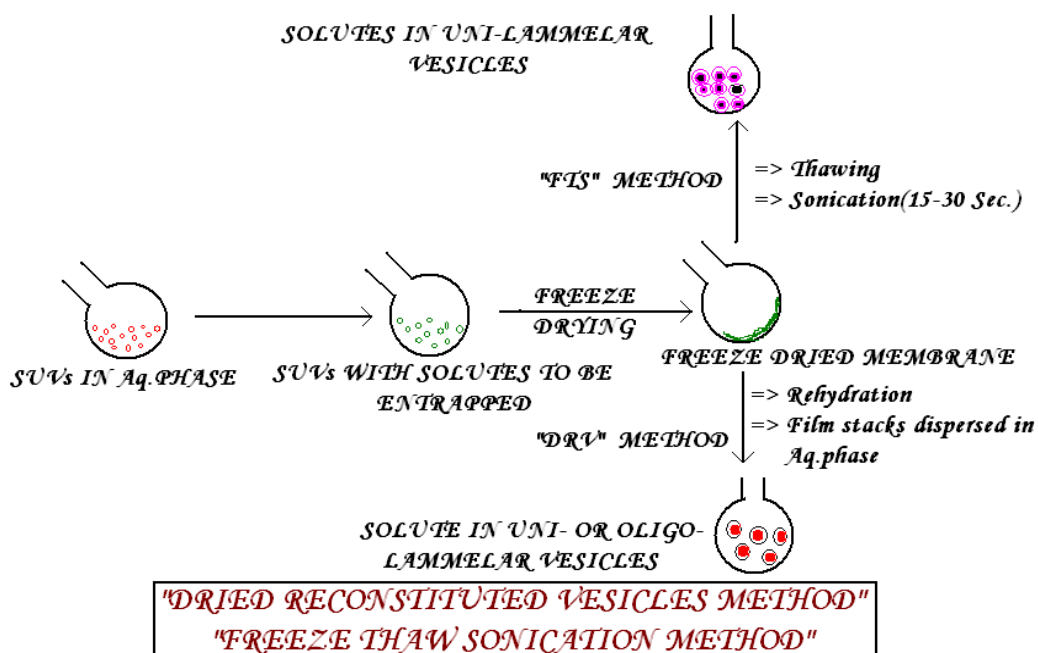


Fig 8. Method of Preparation of Liposomes by Freeze Thaw Sonication.

Freeze Drying

Another method of dispersing the lipid in a finally divided form prior to addition of aqueous media is to freeze dry the lipid dissolved in a suitable organic solvent. The solvent usually used is tertiary butanol. All the above methods produce MLVs. These are too large or too heterogeneous. In order to modify the size the prepared MLVs are further processed using the following procedures (Horton K 2003).^[62, 63]

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 be 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.^[64, 65]

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.^[66, 67]

pH Induced Vesiculation

pH induced vesiculation This method is used to transform MLVs to LUVs using a change in the pH of the dispersion thus avoiding the use of Sonication and high-pressure application. Preformed MLVs (Prepared using hand shaking, freeze thawing) pH 2.5-3.0 exposed to high pH i.e., 11.0 less than 2min (1M NaOH) pH reduced by 0.1M HCl until pH 7.5 SUVs dispersion.^[68]

(B) SOLVENT DISPERSION

Ether and Ethanol Injection

Ethanol Injection: - 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.^[7] 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.^[68, 69]

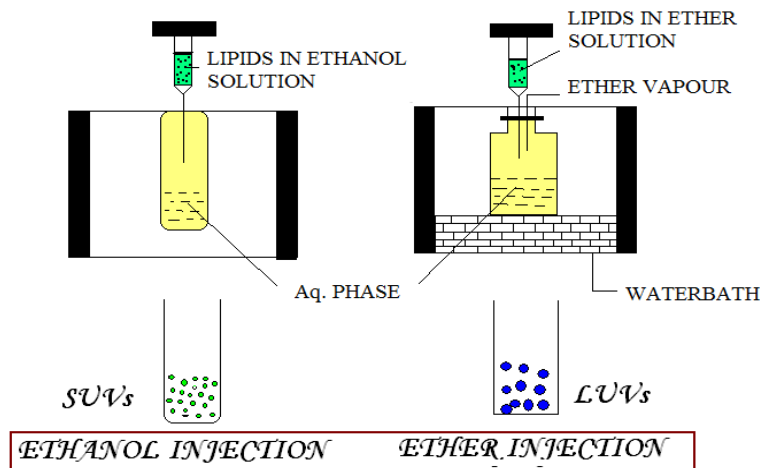


Fig 10. Method of preparation of liposomes by solvent dispersion.

(C) Detergent Removal Method

Solubilisation and Detergent Removal Method

This method is used in the preparation of LUVs and it involves the use of detergent (surfactant) for the solubilisation of the lipids. Detergents used include the non-ionic surfactants e.g., n-octyl-beta-D-glucopyranose (octyl glucide), anionic surfactants (e.g., dodecyl sulphate) and cationic surfactants (e.g., hexadecyltrimethyl ammoniumbromide). The procedure involves the solubilisation of the lipids in an aqueous solution of the detergent and the protein(s) to be encapsulated. The detergent should have a high critical micelle concentration (CMC), so that it is easily removed. The detergent is subsequently removed by dialysis or column chromatography. During detergent removal, LUVs of diameter 0.08–0.2 μm are produced.^[71,72] Detergent/Phospholipids mixtures can form large unilamellar vesicles upon removal of non ionic detergent using appropriate adsorbents for the detergent. In this method, the phospholipid are brought into intimate contact with the aqueous phase via the intermediary of detergent, which associated with phospholipid molecule from water. In structural formed as a result of this associated are known as micelles and can be composed of several hundred component molecules. Their shape and size depend on chemical nature of the detergent, the concentration and other lipid involved. The concentration of detergent in water at which micelles form is known as critical micelle concentration (CMC).^[73,74]

Detergent Solubilization

In this method, phospholipid are brought into intimate contact with the aqueous phase via intermediary of detergents, which associate with the phospholipid molecules and serve to screen the hydrophobic portion of molecule from water. Structure formed as a result of this association is known as micelles and can be composed of several hundred component molecules. Concentration of detergent in water at micelles just start to form is known as CMC (critical micelles concentration).

To remove the detergent and all the transition of mixed micelles to concentric bilayered from 3 methods

Dialysis

Dialysis In contrast to phospholipids, detergents are highly soluble in both aqueous and organic media and there is equilibrium between the detergent molecules in the water phase and in the lipid environment of the micelles. Upon lowering the concentration of the detergent in the bulk aqueous phase, the molecules of the detergents can be removed by dialysis. E.g., of detergent: bile salts sodium cholate and sodium deoxycholate and synthetic detergents such as octylglucoside. Dialysis: Egg PC + sodium cholate (2:1) vesicles(100nm) Trade name-LIPOREP dialysis.

Column Chromatography

Column chromatography Phospholipids + deoxycholate (sonicated vesicles 2:1 or as a dry film) removal of deoxycholate by Column chromatography (Sephadex G-25) Unilamellar vesicles (100 nm).

Detergent Adsorption Using Bio-Beads

Detergent adsorption using bio-beads Detergent (non-ionic)/phospholipids mixtures can form LUVs by removal of non-ionic detergent (Triton X-100) using appropriate adsorbents for the detergent. E.g. Casted lipid film + 0.5-1.0% Triton X-100 + washed bio-beads(0.3g/ml of dispersion) and rocked for about 2hrs at 4 ± 1 °C gives LUVs.^[75,76,77]

2. Active Loading Technique

The utilization of liposomes as drug delivery system is stimulated with the advancement of efficient encapsulation procedures. The membrane from the lipid bilayer is in general impermeable to ions and larger hydrophilic molecules. Ions transport can be regulated by the ionophores while permeation of neutral and weakly hydrophobic molecule can be controlled

by concentration gradients. Some weak acid or bases however, can be transported through the membrane due to various transmembrane gradient, such as electric, ionic (pH) or specific salt (chemical potential) gradient. Several method exist for improved loading of drugs, including remote (active) loading method which load drug molecules into preformed liposome using pH gradient and potential difference across liposomal membrane. A concentration difference in proton concentration across the membrane of liposomes can drive the loading of amphipathic molecule.^[68]

Active loading methods have the following advantages over passive encapsulation

Technique

1. A high encapsulation efficiency and capacity.
2. A reduced leakage of the encapsulated compounds.
3. “Bed side” loading of drugs thus limiting loss of retention of drugs by diffusion, or chemical degradation during storage.
4. Flexibility of constitutive lipid, as drug is loaded after the formation of carrier unit.
5. Avoidance of biological active compounds during preparation step in the dispersion thus reducing safety hazards.
6. The transmembrane pH gradient can be developed using various method. Depending upon the nature of drug to be encapsulated.^[69, 70]

Purification of Liposomes

Liposomes are commonly purified by either gel filtration column chromatography or by dialysis or centrifugation. In column chromatographic separation Sephadex G-50 is most widely used material. In this column chromatographic separation liposome membrane may bind or interact with the surface of the polydextran beads. There may be small amount of lipid lost resulting into destabilization of the membrane leading to permeability changes and subsequent leakage of entrapped solute. This problem can be overcome either by avoiding forming too small size liposomes of the same lipid composition as the test sample either before or after the packing of the column.^[78, 79]

Pharmacokinetics of Liposomes

1. Liposomal drugs can be applied through various routes, but mainly i.v. and topical administration is preferred. After reaching in the systemic circulation or in the local area, a liposome can interact with the cell by any of the following methods.
2. Endocytosis by phagocytotic cells of the R.E.S such as macrophages and Neutrophils.

3. Adsorption to the cell surface either by non specific weak hydrophobic or electrostatic forces or by specific interaction with cell surface components.
4. Fusion with the plasma cell membrane by insertion of lipid bilayer of liposome into plasma membrane with simultaneous release of liposomal contents into the cytoplasm.
5. Transfer of liposomal lipids to cellular or sub cellular membrane or vice versa without any association of the liposome contents.
6. It is often difficult to determine what mechanism is operative and more than.
7. One may operate at the same time.^[80, 81]

Targeting of Liposomes

1) Passive Targeting

As a mean of passive targeting, such usually administered liposomes have been shown to be rapidly cleared from the blood stream and taken up by the RES in liver spleen. Thus capacity of the macrophages can be exploited when liposomes are to be targeted to the macrophages. This has been demonstrated by successful delivery of liposomal antimicrobial agents to macrophages. Liposomes have now been used for targeting of antigens to macrophages as a first step in the index of immunity. For e.g. in rats the i.v. administration of liposomal antigen elicited spleen phagocyte mediated antibody response where as the non liposome associated antigen failed to elicit antibody response.

2) Active Targeting

A pre requisite for targeting is the targeting agents be positioned on the liposomal surface such that the interaction with the target i.e., the receptor is tabulated such as a plug and socket device. The liposome physically prepared such that the lipophilic part of the connector is anchored into the membrane during the formation of the membrane. The hydrophilic part on the surface of the liposome, to which the targeting agent should be held in a sterically correct position to bond to the receptor on the cell surface (Sharma A. 1997).^[81,82,83]

Tab1. Reasons to use liposome as drugs carriers.

S. No.	Properties	Reasons
1	Solubilisation	Liposome may solubilise lipophilic drugs that that would otherwise be difficult to administer intravenously ⁸³
2	Protection	Liposome-encapsulated drugs are inaccessible to metabolizing enzymes; conversely, body components (such as erythrocyte or tissue at the site) are not directly exposed to the full dose of the drug.
3	Duration of action	Liposome can prolong drug action by slowly releasing the drug

		in the body.
4	Direct potential	Targeting options change the distribution of the drug through the body
5	Internalisation	Liposomes are endocytosed or phagocytosed by cell, opening up opportunities to use 'liposome-dependent drugs'. Lipid-based structure (not necessarily liposomes) are also able to bring plasmid material in to cell through the same mechanism (non-viral infection system).
6	Amplification	Liposomes can be used as adjuvants in vaccine formulations.

(D J A Crommelin and H Schreier 1994)^[83, 84, 85]

Factors Affecting

1. Raw Material
2. Manufacturing Process and Controls
3. Storage and Reconstitution

Raw Materials and Their Specifications

1. Lipids From Natural Sources: Egg lecithins can vary in fatty acid composition
2. Modified Natural Lipids
3. Semisynthetic Lipids
4. Synthetic Lipids
5. Examples: Fatty Acid Composition: Degree of Fatty Acid Unsaturation.

Manufacturing Process and Controls

1. Removal of Residual Organic Solvent
2. Removal of Endotoxin
3. Removal of Free Drug
4. Protection of Lipids From Oxidation
5. Control of Liposome Size Distribution / Osmolality
6. Encapsulation Control
7. Sterilization
8. Scale up and Economic Feasibility.

Characterization of Liposomes

Liposome prepared by one of the preceding method must be characterized. The most important parameters of liposome characterization include visual appearance, turbidity, size

distribution, lamellarity, concentration, composition, presence of degradation products, and stability.

1. Visual Appearance

Liposome suspension can range from translucent to milky, depending on the composition and particle size. If the turbidity has a bluish shade this means that particles in the sample are homogeneous; a flat, gray color indicates that presence of a nonliposomal dispersion and is most likely a disperse inverse hexagonal phase or dispersed microcrystallites. An optical microscope (phase contrast) can detect liposome $> 0.3 \mu\text{m}$ and contamination with larger particles.^[94]

2. Determination of Liposomal Size Distribution

Size distribution is normally measured by dynamic light scattering. This method is reliable for liposomes with relatively homogeneous size distribution. A simple but powerful method is gel exclusion chromatography, in which a truly hydrodynamic radius can be detected. Sephacryl-S100 can separate liposome in size range of 30-300nm. Sepharose -4B and -2B columns can separate SUV from micelles.^[95,96]

3. Determination of Lamillarity

The lamellarity of liposomes is measured by electron microscopy or by spectroscopic techniques. Most frequently the nuclear magnetic resonance spectrum of liposome is recorded with and without the addition of a paramagnetic agent that shifts or bleaches the signal of the observed nuclei on the outer surface of liposome. Encapsulation efficiency is measured by encapsulating a hydrophilic marker.

4. Liposome Stability

Liposome stability is a complex issue, and consists of physical, chemical, and biological stability. In the pharmaceutical industry and in drug delivery, shelflife stability is also important. Physical stability indicates mostly the constancy of the size and the ratio of lipid to active agent. The cationic liposomes can be stable at 4°C for a long period of time, if properly sterilized.

5. Entrapped Volume

The entrapped volume of a population of liposome (in $\mu\text{L}/\text{mg}$ phospholipid) can often be deduced from measurements of the total quantity of solute entrapped inside liposome

assuring that the concentration of solute in the aqueous medium inside liposomes is the same after separation from untrapped material. For example, in two phase method of preparation, water can be lost from the internal compartment during the drying down step to remove organic solvent.^[96, 97]

$$\% \text{Entrapment efficiency} = \frac{\text{Entrapped drug (mg)}}{\text{Total Drug Added (mg)}} \times 100$$

6. Surface Charge

Liposome is usually prepared using charge imparting constituting lipids and hence it is imparting to study the charge on the vesicle surface. In general two method are used to assess the charge, namely freeflow electrophoresis and zeta potential measurement. From the mobility of the liposomal dispersion in a suitable buffer, the surface charge on the vesicles.^[98]

Tab2. Physical Characterization.

Characterization parameters	Analytical method/Instrument
Vesicle shape and surface morphology	Transmission electron microscopy, Freeze-fracture electron microscopy
Mean vesicle size and size distribution (submicron and micron range)	Dynamic light scattering, zetasizer, Photon correlation spectroscopy, laser light scattering, gel permeation and gel exclusion
Surface charge	Free-flow electrophoresis
Electrical surface potential and surface pH	Zetapotential measurements & pH sensitive probes
Lamellarity	Small angle X-ray scattering, 31P-NMR, Freeze-fracture electron microscopy
Phase behavior	Freeze-fracture electron microscopy, Differential scanning calorimetry
Percent of free drug/ percent capture	Minicolumn centrifugation, ion-exchange chromatography, radiolabelling

Tab3. Characterization^[98, 99]

Characterization parameters	Analytical method/Instrument
Phospholipid concentration	Barlett assay, Stewart assay, HPLC
Cholesterol concentration	Cholesterol oxidase assay and HPLC
Phospholipid peroxidation	UV absorbance, Iodometric and GLC

Tab 4 .Biological Characterization^[98]

Characterization parameters	Analytical method/Instrument
Sterility	Aerobic or anaerobic cultures
Pyrogenicity	Limulus Amebocyte Lysate (LAL) test
Animal toxicity	Monitoring survival rates, histology and pathology

Stability of Liposomes

1. Physical Stability

The stability of a pharmaceutical product usually is defined as the capacity of the delivery system to remain within defined or pre-established limits during the self life of the product. There is no established protocol for either accelerated or long-term stability studies for the liposomal formulation. Classical models from colloidal science can be used to describe liposome stability. Colloidal systems are stabilized electrostatically, sterically or electrosterically. In addition the selfassembling colloids can undergoes fusion or phase change after aggregation. Liposome exhibit both physical and chemical stability characteristics. Generally, the physical characteristic describes the preservation of liposome structure and the chemical characteristic α -tocopherol.^[100]

Application of Liposome

Therapeutic Application of Liposome

1. Liposome as drug/protein delivery vehicles
 - a) Controlled and sustained drug release.
 - b) Enhanced drug solubilisation.
 - c) Altered pharmacokinetics and biodistribution.
 - d) Enzyme replacement therapy and biodistribution.
 - e) Enzyme replacement therapy and lysosomal storage disorder.

2. Liposome in antimicrobial, antifungal and antiviral therapy
 - a) Liposomal drugs.
 - b) Liposomal biological response modifiers.

3. Liposome in tumour therapy
 - a) Carrier of small cytotoxic molecules.

- b) Vehicle for macromolecules as cytokines or genes.
4. Liposome in gene delivery
- a) Gene and antisense therapy, Genetic (DNA) vaccination.
5. Liposome in immunology
- a) *Immunoadjuvant *Immunomodulator*Immunodiagnosis.
6. Liposome as artificial blood surrogates.
7. Liposome as radiopharmaceutical and radio diagnostic carriers.
8. Liposome in cosmetics and dermatology.
9. Liposome in enzyme immobilization and bioreactor technology.

Tab 5. Applications of Liposomes.

S.No	Discipline	Application
1	Mathmatics	Topology of 2-Dimensional surfaces in 3-Dimensional space governed only by bilayer elasticity
2	Physics	Aggregation behavior, fractals, soft and high-strength material
3	Biophysics	Permeability, Phase transition in 2-Dimension, Photophysics
4	Physical chemistry	Colloid behavior in a system of well-defined physical characteristics, inter- and intra-aggregate forces, DLVO
5	Chemistry	Photochemistry, artificial photosynthesis, catalysis, microcompartmentalization
6	Biochemistry	Reconstitution of membranes, cell function, fusion, recognition studies of drug action
7	Biology	Model biological membranes, Cell function, fusion, recognition
8	Pharmaceutics	Studies of drug action
9	Medicine	Drug-delivery and medical diagnostics, gene therapy

Tab 6. Some liposomal cosmetics formulations currently in market.

S.No.	Product	Manufacturer	Liposomes and key ingredients
1	Capture	Cristian Dior	Liposomes in gel with ingredients
2	Efect du Soleil	L'Oreal	Tanning agents in liposomes
3	Niosomes	Lancome (L'Oreal)	Glyceropolyether with moisturizes
4	Nactosomes	Lancome (L'Oreal)	Vitamins
5	Formule liposome gel	Payot(Fedinand Mushlens)	Thymoxins, hyluronic acid
6	Future Perfect Skin gel	Estee Lauder	TMF, VitaminsE, A Palmitate, cerebroside ceramide, phospholipid
7	Symphatic 2000	Biopharm GmbH	Thymus extract, vitamin A palmitate

8	Natipide II	Nattermann PL	Liposomal gel for do-it-yourself cosmetics
9	Flawless finish	Elizabeth Arden	Liquid make-up
10	Inovita	Pharm/Apotheke	Thymus extract, hyaluronic acid, vitamin E
11	Eye perfector	Avon	Soothing cream to reduce eye irritation
12	Aquasome LA	Nikko Chemical Co.	Liposome with humectants ¹¹⁷

Tab 7. List of marketed products^[118, 119, 120]

Marketed product	Drug used	Target diseases	Company
Doxil TM or Caelyx TM	Doxorubicin	Kaposi's sarcoma	Sequus, usa ¹²⁰
DaunoXome TM	DaunSolid tumours rubicin	Kaposi's sarcoma, breast & lung cancer	Nexstar, USA
Amphotec TM	Amphotericin-B	fungal infections, Leishmaniasis	Sequus, usa ¹¹⁸
Fungizone [®]	Amphotericin-B	fungal infections, Leishmaniasis	Bristol-squibb, Netherland
Ventustm	Prostaglandin-E1	Systemic inflammatory diseases	The liposome company, USA
Alectm	Dry protein free powder of DPPC-PG	Expanding lung diseases in babies	Britannia pharm, uk
Topex-Br	Terbutaline sulphate	Asthma	Ozone, USA
Depocyt	Cytarabine	Cancer therapy	Skye pharm, usa
Novasome [®]	Smallpox vaccine	Smallpox	Novavax, USA
Avian retrovirus vaccine	Killed avian retrovirus	Chicken pox	Vineland lab, USA
Doxil [®]	Doxorubicin Hcl	Refractory ovarian cancer	Alza, usa ¹²⁰

CONCLUSION

Liposomes are acceptable and superior carriers having ability to encapsulate hydrophilic and lipophilic drugs and protect them from degradation. It also has affinity to keratin of horny layer of skin and can penetrate deeper into skin and hence give better absorption. Applied on the skin, liposomes may act as a solublizing matrix for poorly soluble drugs, penetration enhancer as well as local depot at the same time diminishing the side effects of these drugs. The success of liposomes as drug carriers has been reflected in a number of liposome-based formulations, which are commercially available or are currently undergoing clinical trials. The mechanisms giving rise to the therapeutic advantages of liposomes, such as the ability of long-circulating liposomes to preferentially accumulate at disease sites such as tumours, sites of infection and sites of inflammation are increasingly well understood.

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