

LIPOSOME: METHODS OF PREPARATION AND APPLICATIONS

J.S. Dua¹, Prof. A. C. Rana², Dr. A. K. Bhandari³

Address for Correspondence

¹Research Scholar, JNU, Jodhpur and Asst. Professor, Shivalik College of Pharmacy, Nangal, Punjab

²Director, Rayat institute of Pharmacy, Nawanshahr, Panjab

³Jodhpur National University, Jodhpur, Rajasthan

INTRODUCTION

A **liposome** is a tiny bubble (vesicle), made out of the same material as a cell membrane. Liposomes can be filled with drugs, and used to deliver drugs for cancer and other diseases.

Liposomes were first described by British haematologist Dr Alec D Bangham FRS 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. The resemblance to the plasmalemma was obvious, and the microscope pictures served as the first real evidence for the cell membrane being a bilayer lipid structure.

The name liposome is derived from two Greek words: 'Lipos' meaning fat and 'Soma' meaning body. Structurally, liposomes are concentric bleb vesicles in which an aqueous volume is entirely enclosed by a membranous lipid bilayer. Membranes are usually made of phospholipids, which are molecules that have a hydrophilic head group and a hydrophobic tail group. The head is attracted to water, and the tail, which is made of a long hydrocarbon chain, is repelled by water.¹



Fig. - 1: Scheme of a liposome formed by phospholipids in an aqueous solution.

In nature, phospholipids are found in stable membranes composed of two layers (a bilayer). In the presence of water, the heads are attracted to water and line up to form a surface facing the water. The tails are repelled by water, and line up to form a surface away from the water. In a cell, one layer of heads faces outside of the cell, attracted to the water in the environment, and another layer of heads faces inside the cell, attracted by the water inside the cell. The hydrocarbon tails of one layer face the hydrocarbon tails of the other layer, and the combined structure forms a bilayer.¹

When membrane phospholipids are disrupted, they can reassemble themselves into tiny spheres, smaller than a normal cell, either as bilayers or monolayers. The bilayer structures are liposomes. The monolayer structures are called micelles.

The lipids in the plasma membrane are chiefly phospholipids like phosphatidylethanolamine and phosphatidylcholine. Phospholipids are amphiphilic with the hydrocarbon tail of the molecule being hydrophobic; its polar head hydrophilic. As the

plasma membrane faces watery solutions on both sides, its phospholipids accommodate this by forming a phospholipid bilayer with the hydrophobic tails facing each other.

Liposomes can be composed of naturally-derived phospholipids with mixed lipid chains (like egg phosphatidylethanolamine), or of pure surfactant components like DOPE (dioleoylphosphatidylethanolamine). Liposomes, usually but not by definition, contain a core of aqueous solution; lipid spheres that contain no aqueous material are called micelles, however, reverse micelles can be made to encompass an aqueous environment.²

ADVANTAGES

Some of the advantages of liposome are as follows:

- Provides selective passive targeting to tumor tissues (Liposomal doxorubicin).
- Increased efficacy and therapeutic index.
- Increased stability via encapsulation.
- Reduction in toxicity of the encapsulated agents.
- Site avoidance effect.
- Improved pharmacokinetic effects (reduced elimination, increased circulation life times).
- Flexibility to couple with site specific ligands to achieve active targeting.¹

Table No. 1

Trade name	Chemical	Manufacturer
AmbiSome	Amphotericin B	NcXstar Pharmaceuticals
Abelect	Amphotericin B	The Liposome Company
Amphotec	Amphotericin B	Sequus Pharmaceuticals
Doxil	Doxorubicin	Sequus Pharmaceuticals
DaunoXome	Daunorubicin	NcXstar Pharmaceuticals

LIST OF LIPOSOMAL FORMULATIONS IN MARKET

Types of liposomes

Liposomes are classified on the basis of

- Structural parameters
- Method of preparation
- Composition and applications

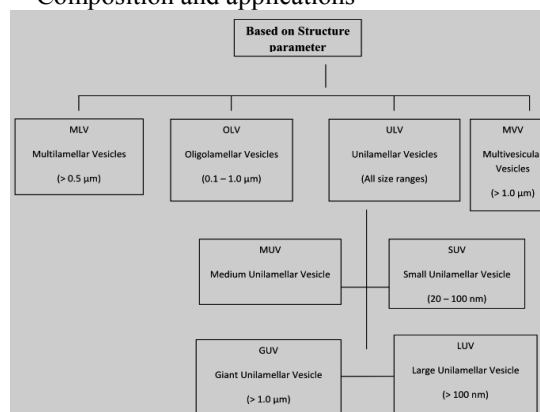


Fig-2: classification of liposomes based on Structural parameters.

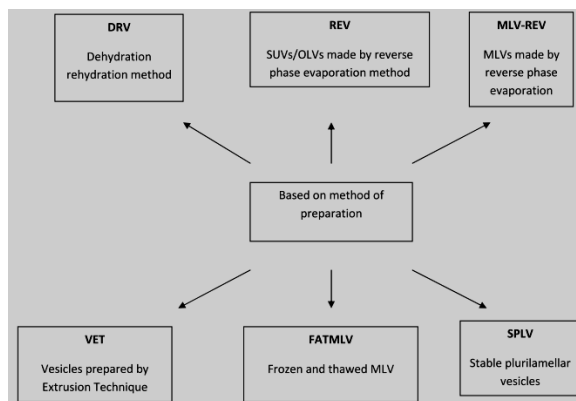


Fig-3: Classification of liposomes based on method of preparation.

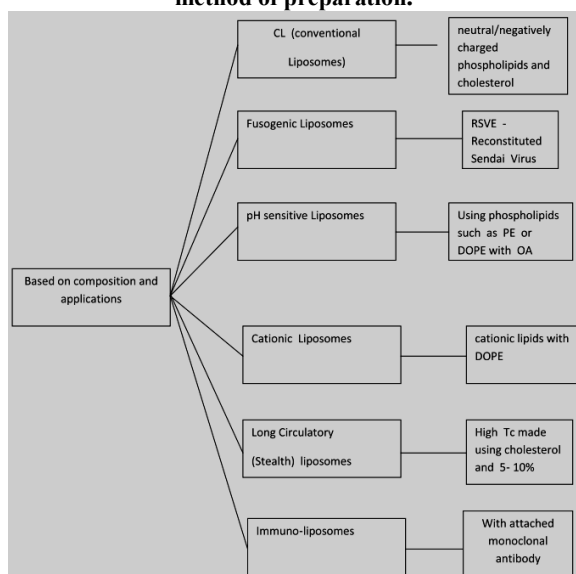


Fig-4: Classification of liposomes based on composition and applications.

Methods of liposomes preparations:

Method:

The correct choice of liposome preparation method depends on the following parameters: 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 lipid vesicles are dispersed; 3) the effective concentration of the entrapped substance and its potential toxicity; 4) additional processes involved during application/delivery of the vesicles; 5) optimum size, polydispersity and shelf-life of the vesicles for the intended application and 6) batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products.^{3,4}

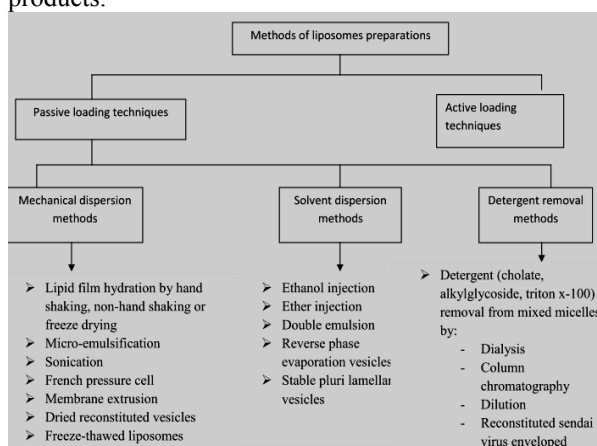


Fig-5: Different methods of liposomes preparations.

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.

GENERAL METHOD OF PREPARATION AND DRUG LOADING

Liposomes are manufactured in majority using various procedures in which the water soluble (hydrophilic) materials are entrapped by using aqueous solution of these materials as hydrating fluid or by the addition of drug/drug solution at some stage during manufacturing of the liposomes. The lipid soluble (lipophilic) materials are solubilized in the organic solution of the constitutive lipid and then evaporated to a dry drug containing lipid film followed by its hydration. These methods involve the loading of the entrapped agents before or during the manufacturing procedure (Passive loading). However, certain type of compounds with ionizable groups, and those which display both lipid and water solubility, can be introduced into the liposomes after the formation of intact vesicles (remote loading).

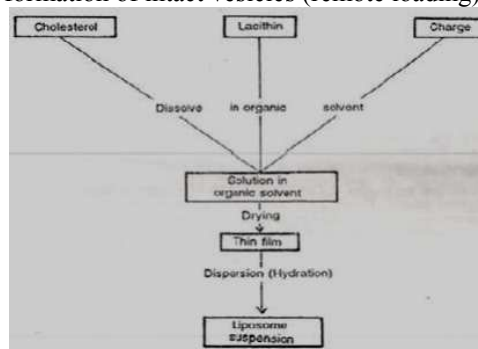


Fig-6: General method of liposomes preparation and drug loading.

MECHANICAL DISPERSION METHODS:

Preparation of liposomes by lipid film hydration:

Preparation of Lipid for Hydration:

When preparing liposomes with mixed lipid composition, the lipids must first be dissolved and mixed in an organic solvent to assure a homogeneous mixture of lipids. Usually this process is carried out using chloroform or chloroform:methanol mixtures. The intent is to obtain a clear lipid solution for complete mixing of lipids. Typically lipid solutions are prepared at 10-20mg lipid/ml of organic solvent, although higher concentrations may be used if the lipid solubility and mixing are acceptable. Once the lipids are thoroughly mixed in the organic solvent, the solvent is removed to yield a lipid film. For small volumes of organic solvent (<1mL), the solvent may be evaporated using a dry nitrogen or argon stream in a fume hood. For larger volumes, the organic solvent should be removed by rotary evaporation yielding a thin lipid film on the sides of a round bottom flask. The lipid film is thoroughly dried to remove residual organic solvent by placing the vial or flask on a vacuum pump overnight. If the use of chloroform is objectionable, an alternative is to dissolve the lipid(s) in tertiary butanol or cyclohexane. The lipid solution

is transferred to containers and frozen by placing the containers on a block of dry ice or swirling the container in a dry ice-acetone or alcohol (ethanol or methanol) bath. Care should be taken when using the bath procedure that the container can withstand sudden temperature changes without cracking. After freezing completely, the frozen lipid cake is placed on a vacuum pump and lyophilized until dry (1-3 days depending on volume). The thickness of the lipid cake should not be more than the diameter of the container being used for lyophilization. Dry lipid films or cakes can be removed from the vacuum pump, the container should be closed tightly and taped, and stored frozen until ready to hydrate.⁵

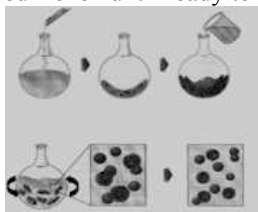


Fig.-7: liposomes prepared by thin layer evaporation technique.

Hydration of Lipid Film/Cake:

Hydration of the dry lipid film/cake is accomplished simply by adding an aqueous medium to the container of dry lipid and agitating. The temperature of the hydrating medium should be above the gel-liquid crystal transition temperature (T_c or T_m) of the lipid. After addition of the hydrating medium, the lipid suspension should be maintained above the T_c during the hydration period. For high transition lipids, this is easily accomplished by transferring the lipid suspension to a round bottom flask and placing the flask on a rotary evaporation system without a vacuum. Spinning the round bottom flask in the warm water bath maintained at a temperature above the T_c of the lipid suspension allows the lipid to hydrate in its fluid phase with adequate agitation. Hydration time may differ slightly among lipid species and structure, however, a hydration time of 1 hour with vigorous shaking, mixing, or stirring is highly recommended. It is also believed that allowing the vesicle suspension to stand overnight (aging) prior to downsizing makes the sizing process easier and improves the homogeneity of the size distribution. Aging is not recommended for high transition lipids as lipid hydrolysis increases with elevated temperatures. The hydration medium is generally determined by the application of the lipid vesicles. Suitable hydration media include distilled water, buffer solutions, saline, and non-electrolytes such as sugar solutions. Generally accepted solutions which meet these conditions are 0.9% saline, 5% dextrose and 10% sucrose. During hydration some lipids form complexes unique to their structure. Highly charged lipids have been observed to form a viscous gel when hydrated with low ionic strength solutions. The problem can be alleviated by addition of salt or by downsizing the lipid suspension. Poorly hydrating lipids such as phosphatidylethanolamine have a tendency to self aggregate upon hydration. Lipid vesicles containing more than 60 mol% phosphatidylethanolamine form particles having a small hydration layer surrounding the vesicle. As particles approach one another there is no hydration repulsion to repel the approaching particle and the

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two membranes fall into an energy well where they adhere and form aggregates. The aggregates settle out of solution as large flocculates which will disperse on agitation but reform upon sitting. The product of hydration is a large, multilamellar vesicle (LMV) analogous in structure to an onion, with each lipid bilayer separated by a water layer. The spacing between lipid layers is dictated by composition with poly-hydrating layers being closer together than highly charged layers which separates on electrostatic repulsion. Once a stable, hydrated LMV suspension has been produced, the particles can be downsized by a variety of techniques, including sonication or extrusion.

Sizing of Lipid Suspension:

Sonication: 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. 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 T_c 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.

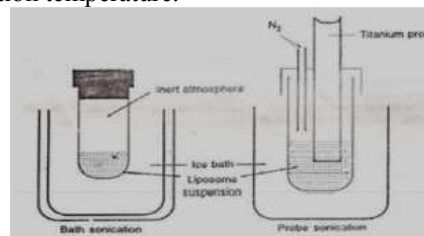


Fig.-8: Sizing of Liposome by sonication

French Pressure Cell Method

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

method are that the temperature is difficult to achieve and the working volumes are relatively small (about 50 mL maximum).⁶

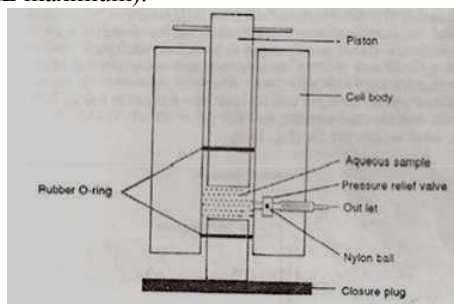


Fig.-9: Liposomes prepared by French Pressure Cell Method

Membrane Extrusion Liposomes:

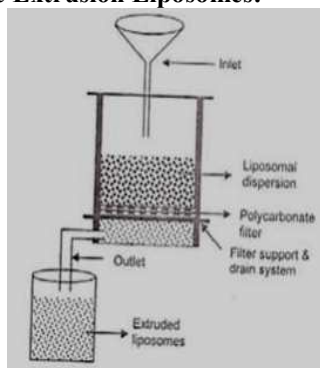


Fig.-10: Liposomes Prepared By Membrane Extrusion Method

Dried Reconstituted Vesicles (DRVs) and Freeze Thaw Sonication (FTS):

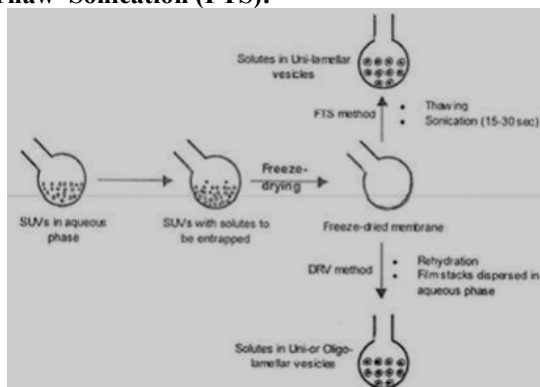


Fig.-11: Liposomes Prepared By Dried Reconstituted Vesicles (DRVs) and Freeze Thaw Sonication (FTS) Method

SOLVENT DISPERSION METHODS

Ether Injection Method

A solution of lipids dissolved in diethyl ether or ether/methanol mixture is slowly injected to an aqueous solution of the material to be encapsulated at 55-65°C or under reduced pressure. The subsequent removal of ether under vacuum leads to the formation of liposomes. The main drawbacks of the method are population is heterogeneous (70-190 nm) and the exposure of compounds to be encapsulated to organic solvents or high temperature.⁷

Ethanol Injection Method

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

macromolecules to inactivation in the presence of even low amounts of ethanol.

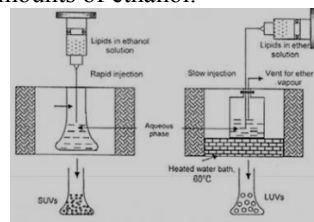


Fig.-12: Liposomes prepared by (a) Ethanol injection method; and (b) Ether injection method

Reverse Phase Evaporation Method

First water in oil emulsion is formed by brief sonication of a two phase system containing phospholipids in organic solvent (diethylether or isopropylether or mixture of isopropyl ether and chloroform) and aqueous buffer. The organic solvents are removed under reduced pressure, resulting in the formation of a viscous gel. The liposomes are formed when residual solvent is removed by continued rotary evaporation under reduced pressure. With this method high encapsulation efficiency up to 65% can be obtained in a medium of low ionic strength for example 0.01M NaCl. The method has been used to encapsulate small and large macromolecules. The main disadvantage of the method is the exposure of the materials to be encapsulated to organic solvents and to brief periods of sonication.⁸

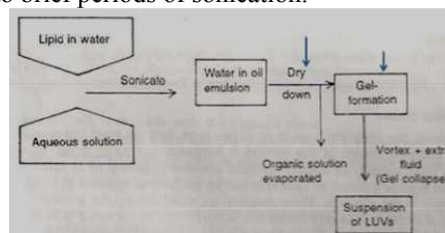


Fig.-13: Liposomes prepared by Reverse phase evaporation method.

DETERGENT REMOVAL METHOD:

The detergents at their critical micelles concentrations have been used to solubilize lipids. As the detergent is removed the micelles become progressively richer in phospholipid and finally combine to form LUVs. The detergents can be removed by dialysis. The advantages of detergent dialysis method are excellent reproducibility and production of liposome populations which are homogenous in size. The main drawback of the method is the retention of traces of detergent(s) within the liposomes. A commercial device called LIPOPREP (Diachema AG, Switzerland) which is a version of dialysis system is available for the removal of detergents. Other techniques have been used for the removal of detergents: (a) by using Gel Chromatography involving a column of Sephadex G-25⁹ (b) by adsorption or binding of Triton X-100 (a detergent) to Bio-Beads SM-2¹⁰ (c) by binding of octyl glucoside (a detergent) to Amberlite XAD-2 beads.¹¹

Industrial production of liposomes

Of the several preparation methods described in the literature, only a few have potential for large scale manufacturing of liposomes. The main issues faced by formulator and production supervisor are presence of organic solvent residues, physical and chemical

stability, pyrogen control, sterility, size and size distribution and batch to batch reproducibility. Liposomes for parenteral use should be sterile and pyrogen free. For animal experiments, adequate sterility can be achieved by the passage of liposomes through 400 nm pore size Millipore filters. For human use, precautions for sterility must be taken during the entire preparation process: that is, (1) the raw materials must be sterile and pyrogen free, (2) preparation in sterile system: working areas equipped with laminar flow and (3) use of sterile containers. Some issues related to phospholipids need attention.^{12,13} The liposomes based on crude egg yolk phospholipids are not very stable. The cost of purified lipids is very high.¹⁴

The liposomes prepared from polymerizable phospholipids are exposed to UV light. The polymerization process takes place in the bilayer(s). Such liposome preparations usually have better storage stability. It should be noted that such materials usually are phospholipid analogues and their metabolic fates have yet to be established.

Detergent Dialysis

A pilot plant under the trade name of LIPOPREFR II-CIS is available from Diachema, AG, Switzerland. The production capacity at higher lipid concentration (80 mg/ml) is 30 ml liposomes/minute. But when lipid concentration is 10-20 mg/ml then up to many liters of liposomes can be produced. In USA, LIPOPREFR is marketed by Dianorm-Geraete.¹⁵

Microfluidization

A method based on microfluidization/ microemulsification/homogenization was developed for the preparation of liposomes. MICROFLUIDIZER is available from Microfluidics Corporation, Massachusetts, USA. A pilot plant based on this technology can produce about 20 gallon/minute of liposomes in 50-200 nm size range. The encapsulation efficiency of up to 75% could be obtained.¹⁴

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

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 manufacturing of liposomes containing particularly lipophilic drugs.¹⁶

Lyophilization

Freeze-drying (lyophilization) involves the removal of water from products in the frozen state at extremely low pressures. The process is generally used to dry products that are thermolabile and would be destroyed by heat-drying. The technique has a great potential as a method to solve long term stability problems with respect to liposomal stability. It is exposed that leakage of entrapped materials may take place during the process of freeze-drying and on reconstitution. Recently, it was shown that liposomes when freeze-dried in the presence of adequate amounts of trehalose (a carbohydrate commonly found at high concentrations in organism)

retained as much as 100% of their original contents. It shows that trehalose is an excellent cryoprotectant (freeze-protectant) for liposomes. Freeze-driers range in size from small laboratory models to large industrial units are available from Pharmaceutical Equipment Suppliers. Conventional liposomes are typically composed of only phospholipids (neutral and/or negatively charged) and/or cholesterol. These are characterized by a relatively short blood circulation time. To overcome this problem, long-circulating liposomes (also called stealth or sterically stabilized liposomes) have been developed. These stealth liposomes carry a polymer coating to obtain prolonged circulation times. Targeted liposomes (immunoliposomes) may be either conventionally or sterically stabilized and have specific antibodies or antibody fragments on their surface to enhance target site binding. Cationic liposomes are still under development for improving the delivery of genetic material. A number of products based on liposomes have already been approved for marketing (see Table no.1), and more are awaiting approval.

Companies such as ADD Drug Delivery Technologies AG (Switzerland), DepoTech Corporation (USA), Nexstar Pharmaceuticals (USA), Novavax Inc. (USA), The Liposome Company Inc. (USA) and Sequus Pharmaceuticals Inc. (USA) are actively involved in the development of liposomal-based drug delivery systems.^{17,18,19}

Table No. 2

Trade name	Chemical name	Manufacture
Ambisome	Amphotericin-B	Gilead sciences
Dauno xome	Daunorubicin	Gilead sciences
Myocet	Doxorubicin	Zeneus
DepoDur	Morphine	Bedminter USA
Depocyt	cytarebine	Enzon pharmaceuticals
Visudyne	Vertporfin	Otho Biotec
Estrasorb	Estradiol	Nova ax
Alveofact	Bovactan	Abbott Laboratories
Abraxane	Paclitaxel	Abraxis Bioscience

Some of the commercially available liposomal/lipid-based products

Current Status of Development:

Anti-cancer drugs such as Doxorubicin (Doxil), Camptothecin and Daunorubicin (Daunoxome) are currently being marketed in liposome delivery systems. Hydroxurea is currently available in i.v. solutions.

Current liposomal drug preparations:

Type of Agents	Examples
Anticancer Drugs	Duanorubicin, Doxorubicin*, Epirubicin, Methotrexate, Cisplatin*, Cytarabin
Anti bacterial	Triclosan, Clindamycin hydrochloride, Ampicillin, peperacillin, rifamicin
Radionuclide	In-111*, Tc-99m
Fungicides	Amphotericin-B*
Vaccines	Malaria merozoite, Malaria sporozoite, Hepatitis B antigen, Rabies virus glycoprotein
Antiviral	AZT
DNA material	cDNA - CFTR*
Enzymes	Hexosaminidase A, Glucocerebrosidase, Peroxidase

*Currently in Clinical Trials or Approved for Clinical Use

Applications of liposome²¹

Liposomes are used for drug delivery due to their unique properties. A liposome encapsulates a region on aqueous solution inside a hydrophobic membrane; dissolved hydrophilic solutes cannot readily pass through the lipids. Hydrophobic chemicals can be dissolved into the membrane, and in this way liposome can carry both hydrophobic molecules and hydrophilic molecules. To deliver the molecules to

sites of action, the lipid bilayer can fuse with other bilayers such as the cell membrane, thus delivering the liposome contents. By making liposomes in a solution of DNA or drugs (which would normally be unable to diffuse through the membrane) they can be (indiscriminately) delivered past the lipid bilayer. There are three types of liposomes - **MLV** (multilamellar vesicles) **SUV** (Small Unilamellar Vesicles) and **LUV** (Large Unilamellar Vesicles). These are used to deliver different types of drugs.²⁰ Liposomes are used as models for artificial cells. Liposomes can also be designed to deliver drugs in other ways. Liposomes that contain low (or high) pH can be constructed such that dissolved aqueous drugs will be charged in solution. As the pH naturally neutralizes within the liposome (protons can pass through some membranes), the drug will also be neutralized, allowing it to freely pass through a membrane. These liposomes work to deliver drug by diffusion rather than by direct cell fusion. Another strategy for liposome drug delivery is to target endocytosis events. Liposomes can be made in a particular size range that makes them viable targets for natural macrophage phagocytosis. These liposomes may be digested while in the macrophage's phagosome, thus releasing its drug. Liposomes can also be decorated with opsonins and ligands to activate endocytosis in other cell types.

- The use of liposomes for transformation or transfection of DNA into a host cell is known as lipofection.
- In addition to gene and drug delivery applications, liposomes can be used as carriers for the delivery of dyes to textiles, pesticides to plants, enzymes and nutritional supplements to foods and cosmetics to the skin.

The use of liposomes in nano cosmetology also has many benefits, including improved penetration and diffusion of active ingredients, selective transport of active ingredients; longer release time, greater stability of active ingredients, reduction of unwanted side effects and high biocompatibility.

Another interesting property of liposomes is their natural ability to target cancer. The endothelial wall of all healthy human blood vessels is encapsulated by endothelial cells that are bound together by tight junctions. These tight junctions stop any large particle in the blood from leaking out of the vessel. Tumour vessels do not contain the same level of seal between cells and are diagnostically *leaky*. This ability is known as the Enhanced Permeability and Retention effect. Liposomes of certain sizes, typically less than 400nm, can rapidly enter tumour sites from the blood, but are kept in the bloodstream by the endothelial wall in healthy tissue vasculature.

Both hydrophilic and hydrophobic drugs can be encapsulated in liposomes. Liposomes are also relatively non-toxic and biodegradable. They therefore have a wide range of biomedical applications.

Protection against enzyme degradation of drugs

Liposomes are used to protect the entrapped drug against enzymatic degradation whilst in circulation. The basis is that the lipids used in their formulation are not susceptible to enzymatic degradation; the

entrapped drug is thus protected while the lipid vesicles are in circulation in the extracellular fluid.

Drug targeting

The approach for drug targeting via liposomes involves the use of ligands (e.g., antibodies, sugar residues, apoproteins or hormones), which are tagged on the lipid vesicles. The ligand recognises specific receptor sites and, thus, causes the lipid vesicles to concentrate at such target sites. By this approach the otherwise preferential distribution of liposomes into the reticuloendothelial system RES (liver, spleen and bone marrow) is averted or minimized.

Topical drug delivery

The application of liposomes on the skin surface has been proven to be effective in drug delivery into the skin. Liposomes increase the permeability of skin for various entrapped drugs and at the same time diminish the side effect of these drugs because lower doses are now required.

Treatment of human immunodeficiency virus (HIV) infections

Several antiretroviral nucleotide analogues have been developed for the treatment of patients suffering from the acquired immunodeficiency syndromes (AIDS). These include antisense oligonucleotide, which is a new antiviral agent that has shown potential therapeutic application against HIV-1.

Enhanced antimicrobial efficacy/ safety

Antimicrobial agents have been encapsulated in liposomes for two reasons. First, they protect the entrapped drug against enzymatic degradation. For instance, the penicillins and cephalosporin are sensitive to the degradative action of β -lactamase, which is produced by certain microorganisms. Secondly, the lipid nature of the vesicles promotes enhanced cellular uptake of the antibiotics into the microorganisms, thus reducing the effective dose and the incidence of toxicity as exemplified by the liposomal formulation of amphotericin B.

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