Liposome: a Powerful Approach for Vesicular Drug Delivery System

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ARTICLE INFO

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.

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 coworkersand was defined as microscopic spherical vesicles that form when phospholipids are hydrated or exposed to anaqueous 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 manufacturing in different lipid compositions or by different method show variation in par. Size , size distribution, surface electrical potential, number of lamella, encapsulation efficacy, Surface modification showed great advantage to produce liposomes of different mechanisims, kinetic properties and biodistribution.[5]

Weiner et aldefined liposome as a microstructure consisting of one or more concentric spheres of lipid bilayer separated by water or aqueous buffer compartments. The typical characteristic of bilayerforming lipids is their amphiphilic nature: a polar head group covalently attached to one or two hydrophobic hydrocarbon tails.[6]When these lipids, e.g., phosphatidylcholine, phosphatidyl ethanolamine or phosphatidyl glycerol, are exposed to an aqueous environment, interactions between themselves (hydrophilic interactions between polar head groups and van der Waals interactions between hydrocarbon chains and hydrogen bonding with water molecules) lead to spontaneous formation of closed bilayers.[7,8]
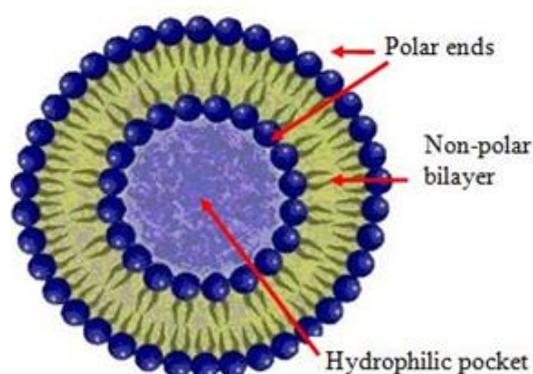

Fig 1. Structure of liposomes

Advantage and disadvantages of liposome

Advantages of Liposome

1. Liposome are increased efficacy and therapeutic index of drug (Actinomycin D).
2. Liposome is increased stability via encapsulation.
3. Liposomes are biocompatible, completely biodegradable, non-toxic, flexible and non immunogenic for systemic and non-systemic administrations.
4. Liposome are reduction in toxicity of the encapsulated agent (Amphotericin B, Taxol).
5. Liposomes help to reduce exposure of sensitive tissues to toxic drugs.
6. Site avoidance effect.

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8. Biocompatible, completely biodegradable, non-toxic, flexible and non immunogenic.

9. Liposomes supply both a lipophilic environment and aqueous "milieu interne" in one system.

10. Liposomes have the ability to protect their encapsulated drug from the external environment.

11. Alter the pharmacokinetic and pharmacodynamic property of drugs (reduced elimination, increased circulation life time).

12. Flexibility to couple with site-specific ligands to achieve active targeting (Anticancer and Antimicrobial drugs).

13. Liposomes can be formulated into multiple dosage forms.

14. Liposomes can encapsulate both micro and macromolecules such as haemoglobin, erythropoeitin, interferon g etc .

15. Provides selective passive targeting to tumour tissue (liposomal doxorubicin).[8, 9, 10]

Disadvantage[9,10]

• Production cost is high.

• Leakage and fusion of encapsulated drug / molecules.

• Sometimes phospholipid undergoes oxidation and hydrolysis like reaction.

• Short half-life.

• Low solubility.

• Fewer stables. The development of liposomes at industrial level is difficult due to its physiological and physicochemical instability.

• They are prone to degradation by oxidation and hydrolysis.

• Production cost is high.

• Leakage and fusion of encapsulated drug / molecules.

• Low solubility

• Fewer stable

Composition of liposomes

Phospholipids

Phospholipids are the major structural component of biological membranes, where two type of phospholipids exit-phospho diglycerides 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 plannar 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:[12,13,14]

1. Phosphatidyl choline (Lecithin) (PC)
2. Phosphatidyl ethanolamine (Cephalin) (PE)
3. Phosphatidyl serine (PS)
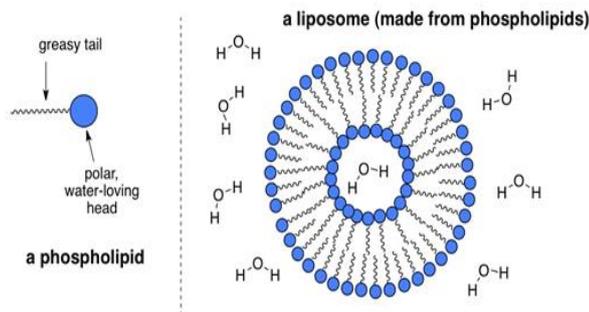4. Phosphatidyl inositol (PI)
5. Phosphatidyl Glycerol (PG)

Fig 2. Structure of liposomes made from phospholipids

Examples of Synthetic phospholipids used in the liposomes are:
1. Dioleoyl phosphatidylcholine (DOPC)
2. Disteroyl phosphatidylcholine (DSPC)
3. Dioleoyl phosphatidylethanolamine (DOPE)
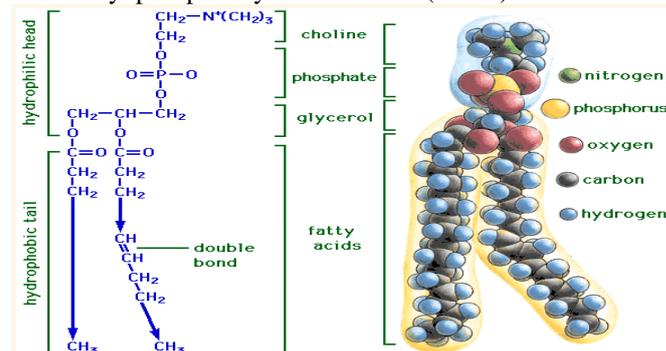4. Distearoyl phosphatidylethanolamine (DSPE)

Fig 3.Structure of Phospholipids

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 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.[13,14,15]
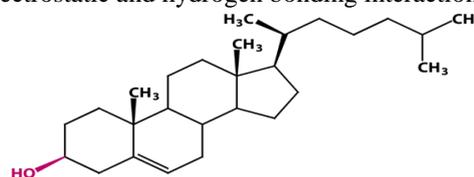

Fig4. Structure of Cholesterol

Mechanism of liposome formation

➢ As liposomes are made up of phospholipids, they are amhpipathic in nature and have ability to binds both aqueous and polar moiety.They have polar head and non polar tail.

➢ The polar end is mainly phosphoric acid and it will bound to water soluble molecule.

➢ 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.

➢ 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.

➢ 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]
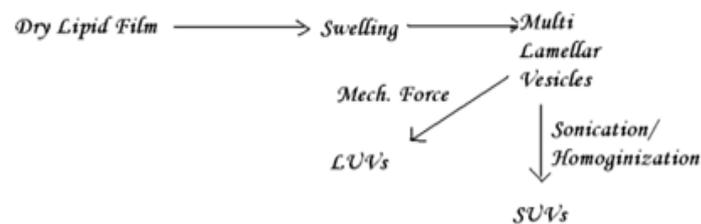

Fig 5. Mechanism of liposome preparation[18]

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 ovolecithin. 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 hencesusceptible to oxidation.Also volatile solvents such as chloroform which are used will tend to evaporate fromthe containe Thus liposomes must be stored in an inertatmosphere 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 nano constructs (artificial membranes) referred to as liposomes, hexasomes 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]
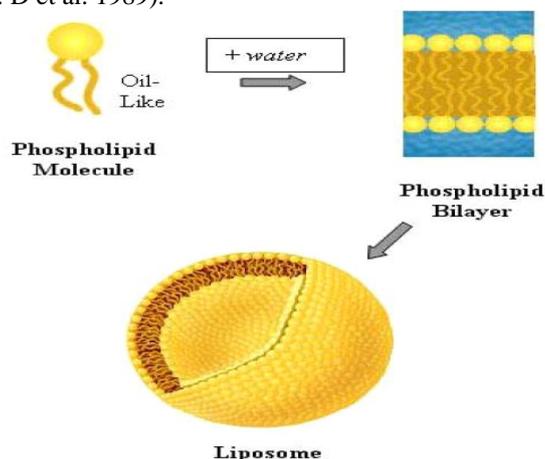
Fig 6. Mechanism of Liposome Formation[31]
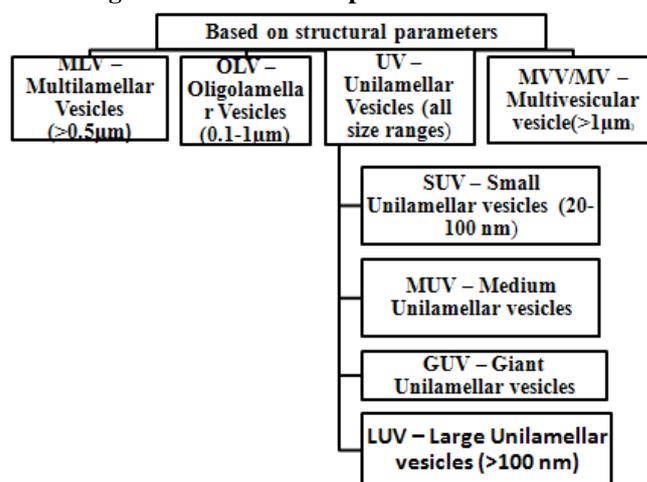
Fig 7. Classification of Liposomes Base On Structural Parameters

Types of Liposomes

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]

Fusogenic liposomes for intracellular delivery

Fusogenic Iiposomes 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 1OOO-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 immuno liposome 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 immuno liposomes 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 immuno liposomes after intravenous administration by coating with PEG, thus giving them a greater chance to reach target sites other than MPS macrophages .[52,53]
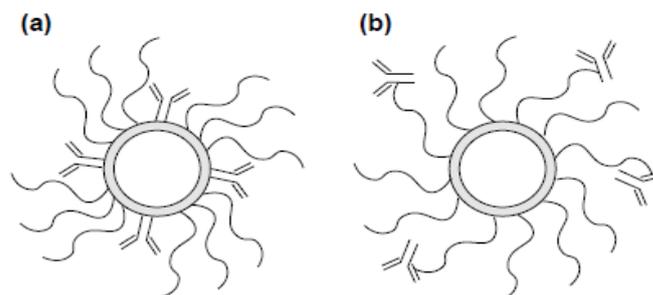
Fig 8. Immobilization of antibody on PEG-liposomes by (a) direct coupling to the liposome surface (which may provide steric hindrance to antigen binding) and (b) copling to the terminal end of PEG chains (which does not give a steric hindrance problem) [52,53]

Method of preparation based on method of dispersion

1. Mechanical dispersion
2. Solvent dispersion methods
3. Detergent removal methods

Physical dispersion or mechanical dispersion method

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

component of the memberane. 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 orgnic solvent are evaproted at about 30 degree Celsius or above the transition temperature of the lipids usedThe evaprotor is isolated from the vaccume source by closing the tep.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 out side the flask.The flask is then removed from the evaproter and fixed on to the manifold of lyophilizer to remove residual solvents.[54,55]

Hydration of lipid layer

After releasing the vacume 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 N_2)and rotated at room temperature and pressure at the same speed or below 60 rpm.The lask is left rotating for 30 minutes or unti all lipid has been removed from the wall of the flask and has given homogenous milky –whjite 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 temparature of the lipid in order to complete the sweeling process to give MLVs.[54,55,56]

Non –shaking vesicles

Method described by reeves and dowben in 1996 by which large unicellular vesicles (LUVv) 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 rotationary movements.Solution of lipid in chloroform :methanol mixture is spred over the flat bottom conical flask.[55,56]The solution is evaproted at room temparature by flow of nitrogen through the flask without disturbing the solutionAfter drying water saturated nitrogen is passed through the flask untill 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 ,10-20ml of 0.2 sucrose in distilled water (degassed)is introduced down the slide 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 flaskThe flask is fluhed with nitrogen ,sealed and allowed to stand for 2hours at 37 degrees celsius .take care not to disturb the flask in any way.Aftre swelling ,the vesicles are harvested by swirling the contents ,to yield a milky –suspention.[57]
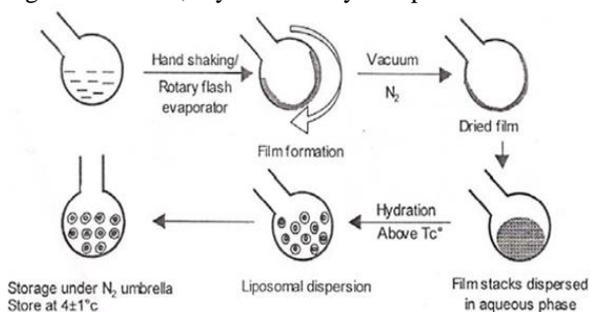
Fig 9. Method of preparation of liposomes by Hand shaking method

Soniction

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 over heating 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]
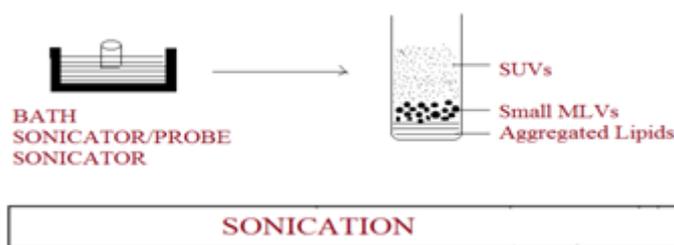
Fig 10. Method of preparation of liposomes by sonication

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. diameterAfter a single pass, the size of vesicles is reduced to a size 0.1 and 0.2um .[58,59]
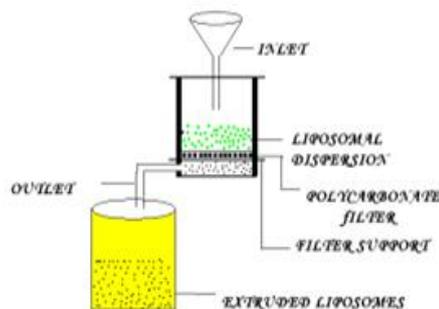
Fig 11. Method of preparation of liposomeby micro emulsification liposomes

Freeze Thaw Sonication

This method is based upon freezing of a unilamellar dispersion(SUV). Then thawing by standing at room temperature for 15min.Finally subjecting to a brief Sonication cycle which considerably reduces the permeability of the liposomes membrane. In order to prepare GIANT VESICLES of diameter between 10 and 50um, the freeze thaw technique has been modified to incorporate a dialysis step against hypo- osmolar buffer in the place of sonication. The method is simple, rapid and mild for entrapped solutes, and results in a high proportion of large unilamellar vesicles formation which are useful for study of membrane transport phenomenon.This method is based upon freezing of a unilamellar dispersion (SUV). Then thawing by standing at room temperature for 15min. Finally subjecting to a brief Sonication cycle which considerably reduces the permeability of the liposomes membrane. In order to prepare GIANT VESICLES of diameter between 10 and 50um, the freeze thaw technique has been modified to incorporate a dialysis step against hypo- osmolar buffer in the place of sonication. The method is simple, rapid and mild for entrapped solutes, and results in a high proportion of large unilamellar vesicles formation which are useful for study of membrane transport phenomenon.[60,61]
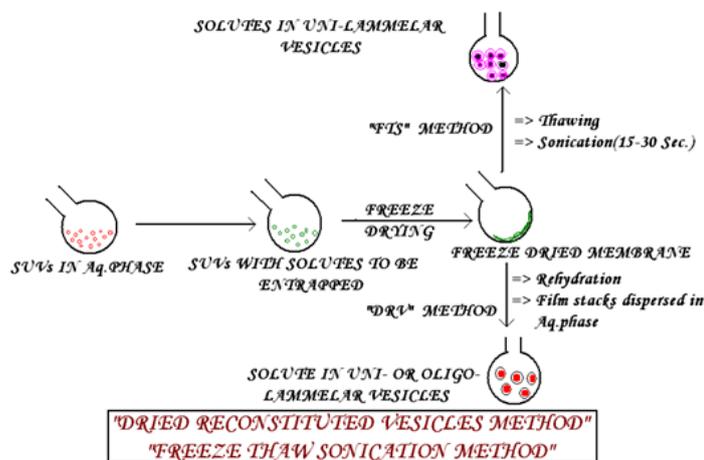

Fig 12. Method of preparation of liposomes by freeze thaw sonication

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 he encapsulated under gentle conditions. The resulting liposomes are largely unilamellar, although of a heterogeneous size range. The chief disadvantage of this method is that LUVs can only be obtained from acidic phospholipids .[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 ie.,11.0 less than 2min (1M NaOH) pH reduced by 0.1M HCl until pH 7.5 SUVs dispersion .[68]

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 issimple 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 MLVsare 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 ethanolbecause it forms azeotrope with water and the possibility of various biologically activemacromolecules to inactivation in the presence of even low amounts of ethanol .[68,69]
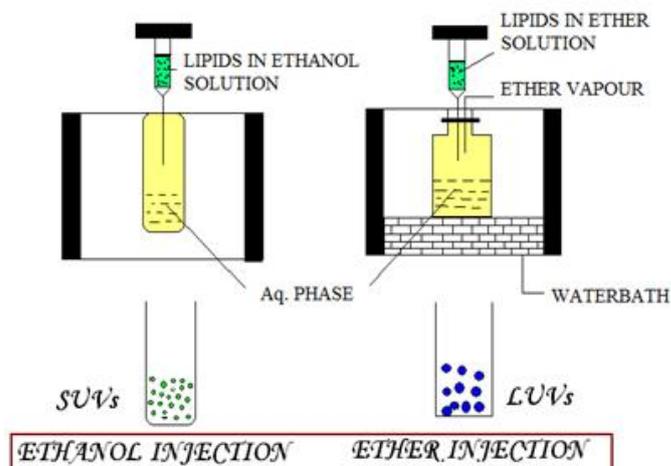
Fig 13. Method of preparation of liposomes by solvent dispersion

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

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-bete- D-glucopyranose (octyl gluside), 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 0.08–0.2µm 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 molucule 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 concentrated of detergent in water at which micelles from is known as critical micelle concentration(CMC).[73,74]

Detergent Solubilization

In this method, phospholipid are brought into intimate contact with the aquous 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 assotiation are 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
• Column chromatography
• Use of biobeads

Dialysis

Dialysis In contrast to phospholipids, detergents are highly soluble in both aqueous and organic media and there is an equilibrium between the detergent molecules n 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]

Purification of Liposomes

Liposomes are commonly purified by either gel filtration column chromatography or by dilysis or centrifugation. In column chromatographic separation Sephadex G-50 is most widely used material. In this column chromatographic separation liposome membrane may bind or interactwith 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 sampleeither before or after the packing of the column. [78,79]

Pharmacokinetics of liposomes
• 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.
• Endocytosis by phagocytotic cells of the R.E.S such as macrophages and
Neutrophils
• Adsorption to the cell surface either by non specific weak hydrophobic or electrostatic forces or by specific interaction with cell surface components
• 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.
• Transfer of liposomal lipids to cellular or sub cellular membrane or vice versa without any association of the liposome contents.
˙ It is often difficult to determine what mechanism is operative and more than one may operate at the same time.[80,81]

Targeting of Liposomes
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.

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 stericaly correct position to bond to the receptor on the cell surface (Sharma A. 1997).[81,82,83]

Factors affecting the formation of liposome

The ultimate identity of any liposomal system and hence its properties are determined by the various factors. All these variables, directly or indirectly, have their effect on the formation of liposomes. Therefore, it is necessary that these variables must be carefully controlled during the design of liposomes. Some of these factors are shown below.[85]
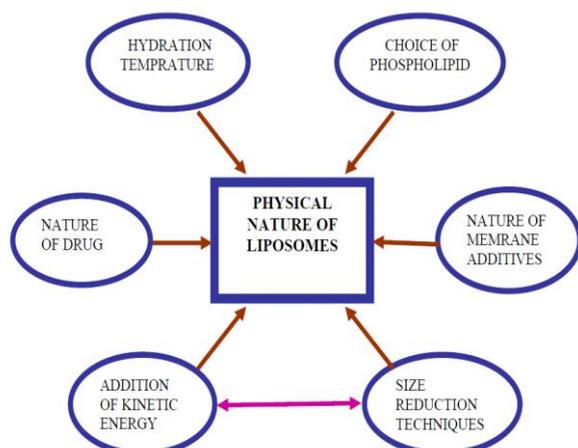

Fig 14. Factors affecting the formation of liposome[85,86]

In other words, during the formulation of liposomes all these variables must be optimized in order to obtain the best possible formulation with maximum stability and entrapment efficiency. The design of drug delivery system should always be taken from the past biology of the system. For example, the anticancer drugs are targeted using liposomes to the specific vascular structure of tumor tissue. Another example includes the use of liposomes to target the drug to liver and spleen in leishmaniasis, as particulate uptake by liver and spleen is a known fact. Once a correlation is obtained between the liposomal surface and the resulting biological response, more specific forms of targeting that involve the incorporation of molecular recognition elements may be undertaken. Stealth liposomes having a coating of polyethylene glycol have been widely exploited for the tumor targeting as they have low uptake by reticulo-endothelial system and spleen (Rajan K. Verma and Sanjay Garg 2001). In the similar manner, the pharmacodynamics and pharmacokinetics of liposomes can be changed by inclusion of charge inducers and steric stabilizers like dicetylphosphate (DCP), stearylamine (SA), solulan C-24 etc.[88,89,90]

Factors affecting
• Raw Material
• Manufacturing Process and Controls
• Storage and Reconstitution

Raw Materials and Their Specifications
• Lipids From Natural Sources: Egg lecithins can vary in fatty acid composition
• Modified Natural Lipids
• Semisynthetic Lipids
• Synthetic Lipids
• Examples:
• Fatty Acid Composition
• Degree of Fatty Acid Unsaturation

Manufacturing Process and Controls
• Removal of Residual Organic Solvent

• Removal of Endotoxin
• Removal of Free Drug
• Protection of Lipids From Oxidation
• Control of Liposome Size Distribution / Osmolality
• Encapsulation Control
• Sterilization
• Scale up and Economic Feasibility

Storage and Reconstitution
• Liposome preparations can be stored:
i. frozen, in liquid form
ii.as a freeze dried powder.
• Reconstitution of freeze-dried liposomes may affect particle size and size distribution (Arthur B. Shaw, Ph.D. 2001).[92,93]

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.

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 μm and contamination with larger particles.[94]

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]

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

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 properlysterilized.

Entrapped Volume

The entrapped volume of a population of liposome (in μL/ 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 unentrapped 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]

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

Table 1. 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-deependent 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]

Table 2. 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 colorimetery
Percent of free drug/ percent capture	Minicolumn centrifugation, ion-exchange chromatography, radiolabelling

Table 3 . Chemical characterization

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

Table 4 . Biological characterization[98, 99]

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

Table 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

Table 6. Applications of Liposomes in basic sciences

Application	Utilized
Liposomes in bioengineering	Modern genetic engineering and gene recombinant technology is based on the deliveryof genetic material, i.e. fragments of DNA, into various cells and microorganismsin order to alter their genetic code and force them to produce particular proteins or Polypeptides[111]
Medical applications of stealth liposomes	Sterically stabilised vesicles can act either as long circulating microreservoirs ortumour (or site of inflammation and infection) targetting vehicles.
Liposomes in anticancer therap	These cells are in tumours, but also in gastrointestinal mucosa, hair, and blood cells and therefore this class of drugs are very toxic. The most used andstudied is Adriamycin (commercial name for Doxorubicin HCl).[114,115]
Macrophage activation and vaccination	The automatic targetting of liposomes to macrophages can be exploited in severalother ways, including the macrophage activation and in vaccination
Liposomes in parasitic diseases and infections	Since conventional liposomes are digested by phagocytic cells in the body after intravenous administration, they are ideal vehicles for the targetting of drug molecules into these macrophages.[112]
Applicationsof liposomes in basic sciences	Lipid membranes are two dimensional surfaces floating in three dimensional spaceIn the simplest models, they can be characterised only by their flexibility whichis related to their bending elasticity.[112,113,114]
Application of liposomes in agro-food industry	The ability of liposomes to solubilize compounds with demanding solubility properties, sequester compounds from potentially harmful milieu, and release incorporated molecules in a sustained and predictable fashion can be used also in the food processing industry For instance, lecithin and some other polar lipids are routinely extracted from nutrients, such as egg yolks or soya beans.[115]

Table 7. 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 Launder	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]

Table 8. List of marketed products[118, 119,120]

Marketed product	Drug used	Target diseases	Company
DoxilTM or CaelyxTM	Doxorubicin	Kaposi's sarcoma	Sequus, usa [120]
DaunoXomeTM	DaunSolid tumoursorubicin	Kaposi's sarcoma, breast & lung cancer	Nexstar, USA
AmphotecTM	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]

Surface Charge

Liposome are 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]

Characterization

Stability of Liposomes

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
• Controlled and sustained drug release
• Enhanced drug solubilization
• Altered pharmacokinetics and biodistribution
• Enzyme replacement therapy and biodistribution
• Enzyme replacement therapy and lysosomal storage disorder
2. Liposome in antimicrobial, antifungal and antiviral therapy
• Liposmal drugs
• Liposomal biological response modifiers
3. Liposome in tumour therapy
• Carrier of small cytotoxic molecules
• Vehicle for macromolecules as cytokines or genes
4. Liposome in gene delivery
Gene and antisense therapy
Genetic (DNA) vaccination
5. Liposome in immunology
• 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 .

Drug targeting

The need for "drug targeting" arises from a problem situation whereby a drug administered (iv for example) enters the blood stream and is distributed to varying extents throughout the body. When the actual desire is to deliver or direct the drug selectively to its site of action. This site could be an organ structure, a cell subset, or even an intracellular region. In such a case pumping the drug throughout the whole body is not only wasteful but, more fundamentally, it is also likely to lead to undesirable side effects. [101,102]On the other hand, restricting the distribution of the drug to the specific target site should allow for an increase in efficacy at low dose with attendant decrease in toxicity. Hence, the benefits of drug targeting include reduced drug waste, and it is possible to deliver a drug to a tissue or cell region not normally accessible to the free or untargeted drug.

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.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 reticuloendeothelial system RES (liver, spleen and bone marrow) is averted or minimized. [103,104]

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. [107,108]

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.[109,110]

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 J-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.

Application of liposomes in cosmetics

The same properties of liposomes can be utilized also in the delivery of ingredients in cosmetics. In addition, liposomes as a carrier itself offers advantages because lipids are well hydrated and can reduce the dryness of the skin which is a primary cause for its ageing. In general the rules for topical drug applications and delivery of other compounds are less stringent than the ones for parenteral administration and several hundred cosmetic products are commercially available since Capture (C. Dior) and Niosomes (L'Oreal) were introduced in 1987. [115,116]They range from simple liposome pastes which are used as a replacement for creams, gels, and ointments for do-it-yourself cosmetical products to formulations containing various extracts, moisturizers, antibiotics, and to complex products containing recombinant proteins for wound or sunburn healing. Most of the products are anti-ageing skin creams. Unrinsable sunscreens, long lasting perfumes, hair conditioners, aftershaves and similar products, are also gaining large fractions of the market. Liposomal skin creams already share more than 10% of the over $10 billion market (Lasic D.D, 1995).[117]

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 suchas tumours, sites of infection and sites of inflammation are increasingly well understood.

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