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REVIEW ARTICLE

Review on Preparation and Characterization of Liposomes with Application

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ABSTRACT

Liposomes are microscopic vesicles composed of a bilayer of phospholipids or any similar amphipathic lipids. They can encapsulate and effectively deliver both hydrophilic and lipophilic substances 2-3 and may be used as a non-toxic vehicle for insoluble drugs. 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 players. Liposomes can be manufactured in different lipid compositions or by different method show variation in particle size, size distribution, surface electrical potential, no. of lamella, encapsulation efficacy, Surface modification showed g+reat advantage to produce Liposomes of different mechanisims, kinetic properties and biodistribution. Products in the market are Doxorubicin (Doxil, Myocet), Daunorubicin (Dauno Xome), Cytarabin (Depocyte), (lymphotmatos meningitis) and Amphotericine B (Ambisome), (fungal infection). 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. Liposomes are nano size artificial vesicles of spherical shape.

Keywords: Liposomes, Microscopic, Phospholipids, Dispersion, Encapsulation.

INTRODUCTION

Liposome was found by Alec Bangham of Babraham Institute in Cambridge, England in 1965. In 1990; drugs with liposome and Amphotericin B were approved by Ireland. In 1995 America F.D.A approved liposor doxodubicin. 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. Considerable progress was made in 1980s, in engineering Liposomes to circulate longer in the blood and

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remain intact while doing so.[1] 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. 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.[2] They can be produced from natural phospholipids and cholesterol. Phospholipids combine with water immediately forms a bi-layered sphere.[3] On this account Dr, Baumann cosmetics produce only liposome products without perfume and without chemical preservatives. Exactly the same phospholipids which comprise the liposome membrane form the walls of skin cells. Similarly, the intercellular substance which is found between the skin cells is composed of phospholipids, ceramides, triglyceride, free fatty acids, cholesterol and water. If skin cells are slightly damaged or if the intracellular substance is lost through aggressive cleansing methods, Liposomes are able to perfectly replenish the missing lipids.

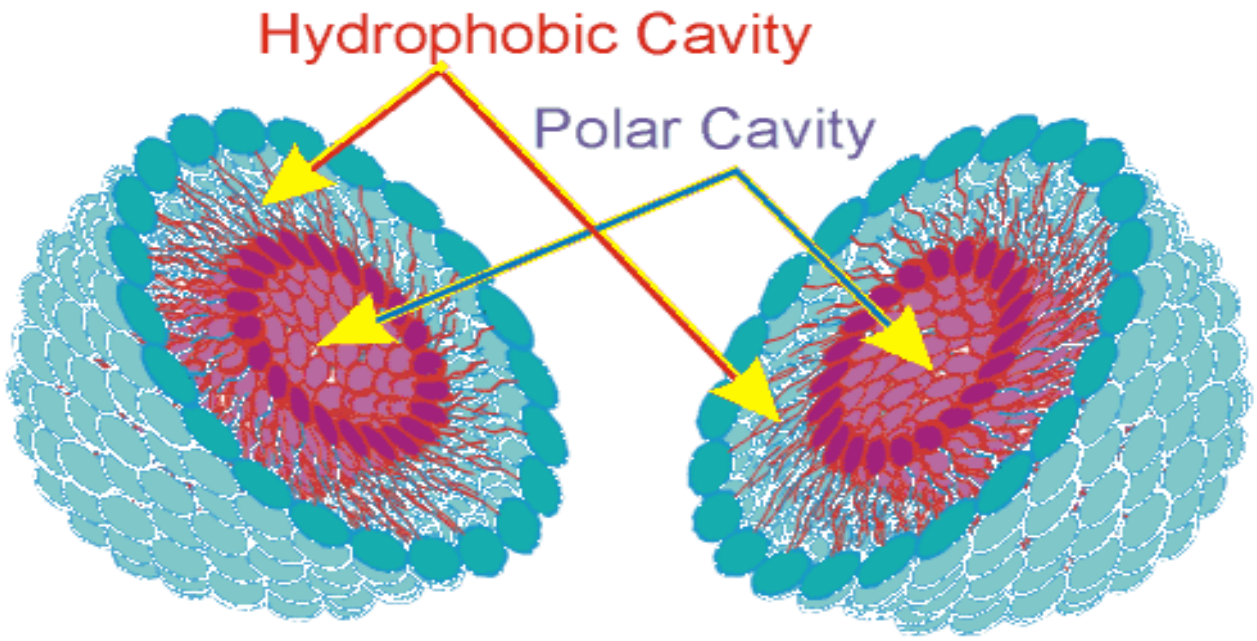

Figure 1: Structure of Liposome

Classification:

Liposomes are classified on the basis of:

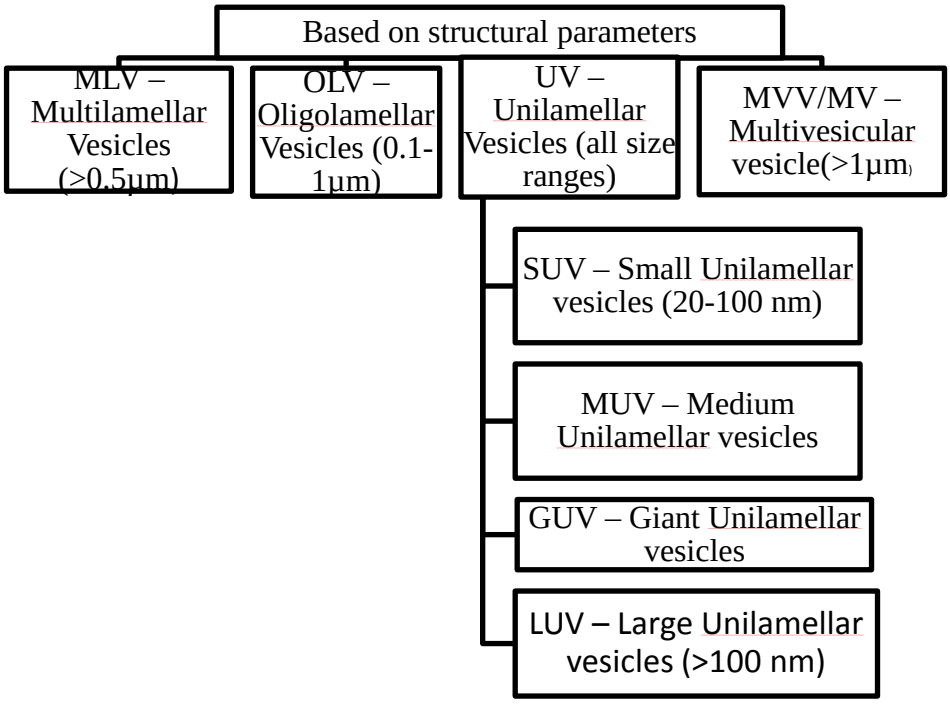

Figure 2: Classification of Liposome

Figure 3: Multilamellar Liposome

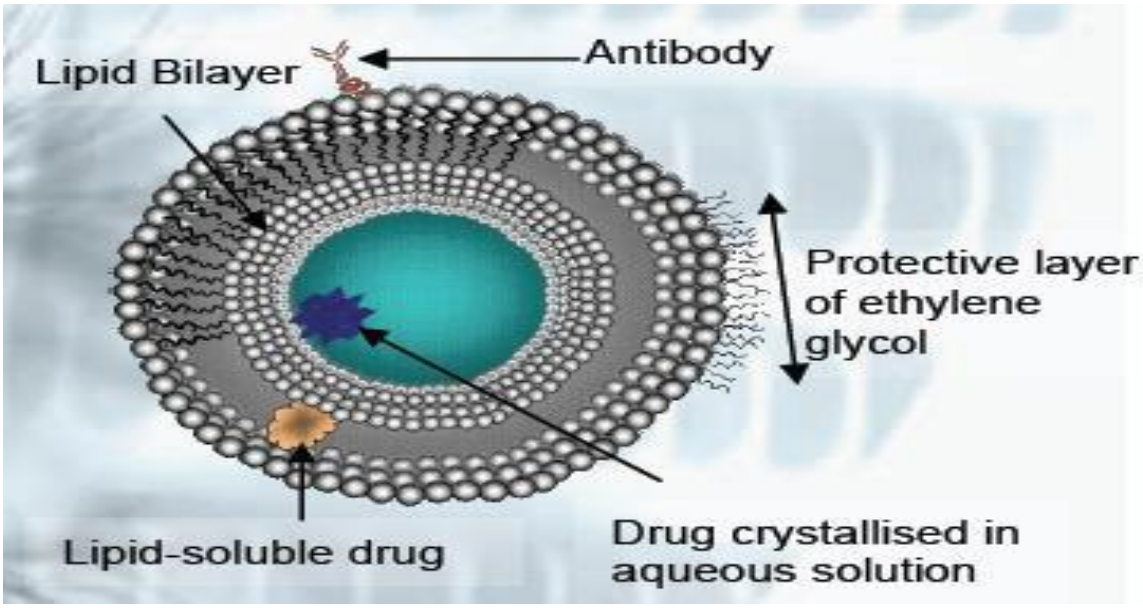

Figure 4: Unilamellar Liposome

Attractive biological properties of Liposomes

- Liposomes are biocompatible.
- Liposomes provide a unique opportunity to deliver pharmaceuticals into cells or even inside individual cellular compartments.
- Size, charge and surface properties of Liposomes can be easily changed simply by adding new ingredients to the lipid mixture before liposome preparation

Lamella:

A Lamella is a flat plate like structure that appears during the formation of Liposomes. The Phospholipid bilayer first exists as a lamella before getting converted into spheres. A Lamella is a flat plate like structure that appears during the formation of Liposomes.

Advantage

- Flexibility in the structure in entrapment of water soluble as well as insoluble drugs.
- Biodegradability
- Efficient control of release.
- Resemblance to natural membrane structures.
- Increased targeting prospects. Biocompatible, completely biodegradable, non-toxic, flexible, nonimmunogenic.
- Liposomes supply both a lipophilic environment and aqueous “milieu internee” in one system. Can protect the encapsulated drug.

- Reduce exposure of sensitive tissues to toxic drugs. .
- Easy for construction.
- Increased efficacy and therapeutic index.
- Provides both targeting active and passive.
- Does not accumulate in heart and so there is no cardiotoxicity.
- Prevent oxidation of the drug.

Disadvantage

- The 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.
- The production cost is high.
- Leakage and fusion of encapsulated drug / molecules.
- Low solubility
- Fewer stables [4]

Composition of Liposomes

A. Phospholipids

Naturally occurring phospholipids used in Liposomes-

1. Phosphatidylcholine

2. Phosphatidylethanolamine

3. Phsphatidylserine

Synthetic phospholipids used in the Liposomes are-

1. Disloyal phosphatidylcholine

2. Destroy phosphatidylcholine

3. Disloyal phosphatidylethanolamine

B. Cholesterol

Cholesterol can be incorporated into phospholipids membrane in very high concentration up to 1:1 or 2:1 molar ratios of cholesterol to phospatidylcholine. Being an amphipathic molecule, cholesterol inserts into the membrane with its hydroxyl group of cholesterol oriented towards the aqueous surface and aliphatic chain aligned parallel to the alkyl chains in the center of the players and also it increase the separation between choline head groups and eliminates the normal electrostatic and hydrogen bonding interaction. The phospholipids are arranged in such a way that the hydrophilic head is exposed outside and the lipophillic tails are aliened inside. This makes the Liposomes water soluble molecules.[5]

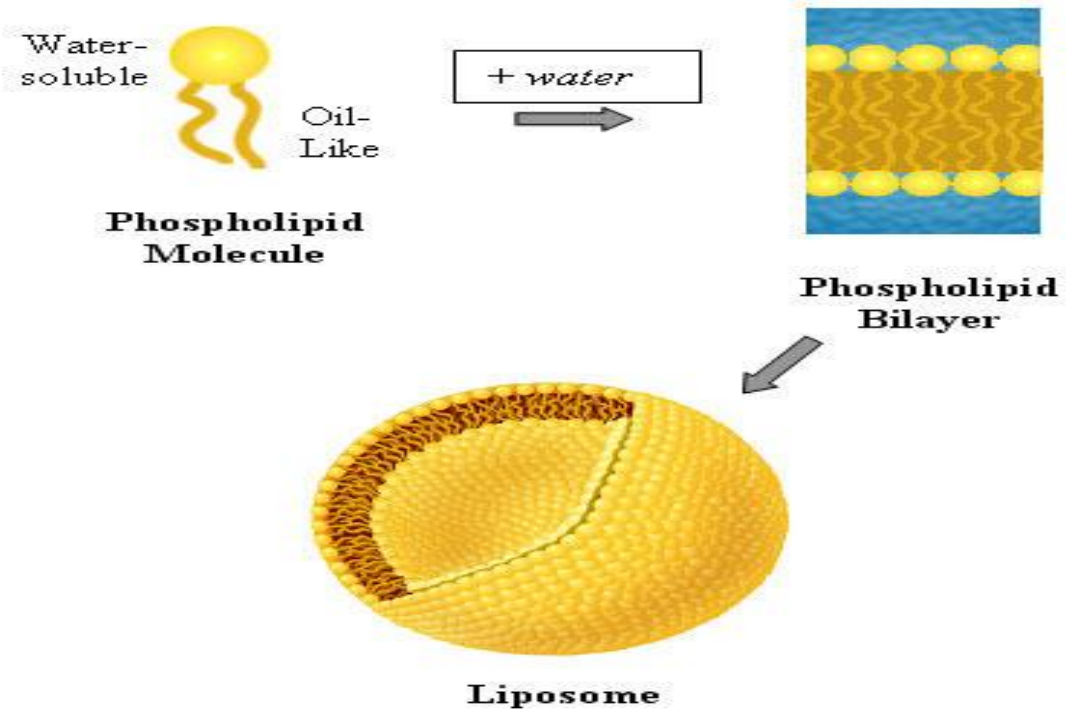

Figure 5: Composition of Liposomes

2. Mechanism: - Liposome Formation

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

The polar end is mainly phosphoric acid and it will bind to water soluble molecules.

In aqueous medium the molecules in self-assembled structure is oriented in such way that the pole portion of the molecule remains 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 players become fluid and swells.

Once these vesicles 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.

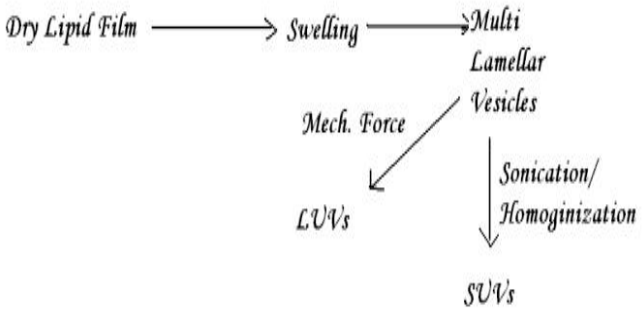

Figure 6: Mechanism of Liposomes

Handling of Liposomes

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

Drying

An important step involved in the preparation ofLiposomes is the drying of the lipid.Large volume of organic solution of lipids is mosteasily dried in a rotary evaporator fitted with acooling coil and a thermostatically controlledwater bath.Rapid evaporation of solvent is carried out bygentle warming (20-40 degrees) under reducedpressure (400-700 mm Hg)Rapid rotation of the solvent containing flaskincreases the surface area for evaporationIn cases where sufficient vacuum is not attainableor if the concentration of lipids is particularlyhigh, it may be difficult to remove the last tracesof chloroform from the lipid film.Therefore, it is recommended as a matter ofroutine that after rotary evaporation,somefurther means is employed to bring the residue tocomplete dryness.Attachment of the flask to the manifold oflyophilizer, and overnight exposure to high vacuum is a good method.[6,7]

3. Method of preparation based on the method of dispersion:

•Mechanical dispersion

•Solvent dispersion methods

•Detergent removal methods

Mechanical dispersion methods types of modified vesicles

•Lipids film hydration by hand shaking, non hand shaking and freeze drying

•Sonication unicellular Liposomes

•Micro-emulsification Liposomes

•French Pressure Cell Liposomes

•Membrane Extrusion Liposomes

•Dried reconstituted vesicles

•Freeze-thawed Liposomes

•PH induced vesiculation

•Calcium induced fusion

Solvent dispersion methods

•Ethanol injection

•Ether injection

•Double emulsion vesicles

•Reverse phase evaporation vesicles

•Stable plurilamellar vesicles

Detergent removal methods

•Detergent

•Dilution

•Reconstituted Sendai virus enveloped vesicles

Physical dispersion or mechanical dispersion method

Aqueous volume enclosed using this method usually 5-10%, which is a 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:-

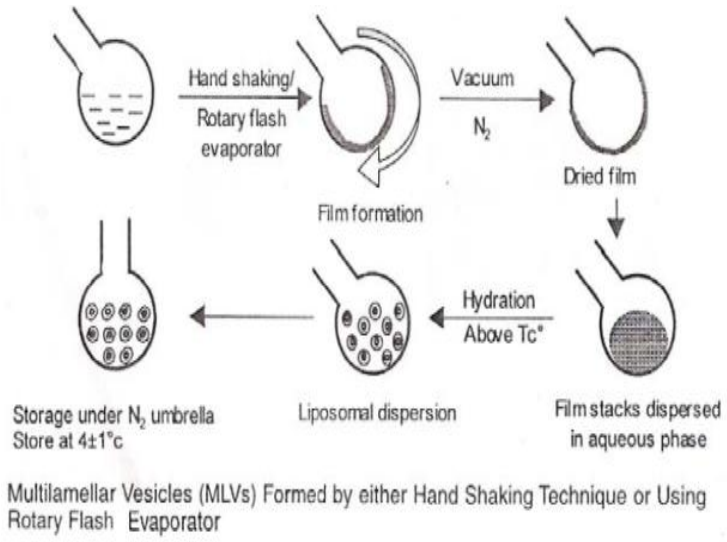

Figure 7: Lipid Film Hydration by hand shaking method

Process in more detail –

Step-1

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 outside the flask. The flask is then removed from the evaproter and

fixed on to the manifold of lyophilizer to remove residual solvents.[8]

Step-2

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 N2) 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.

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. The solution is evaproted at room temparature by flow of nitrogen through the flask without disturbing the solution. After 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 flasks. 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.[8]

Buchi Rotatory Evaporator Non Shaking Method & Pro - Liposomes:

Pro Liposomes method devised to increase the surface of dry lipid while keeping the low aqueous volume In this method, the lipid are dried down to finely divided particulate support, such as powered sodium chloride, or sorbitol or other polysacchride- to give pro-Liposomes. The lipid is swelled upon adding water to dried lipid coated powered (pro-Liposomes), where the support rapidly dissolves to give a suspention of MLVs in aqueous solution. The size of the carrier influences the size and hetrogeneity of the Liposomes. This method overcomes the problem encountered when storing Liposomes themselves in either liquid, dry or frozen form, and ois ideally suited for prparation

where the material to be entrapped incoropted into lipid membrane. In case where 100% entrapment of aqueous component is not essential, this method is also of valve. For preparing pro –Liposomes special equipment i.e. buchi rotary evaproter R'with water cooled condensor coil and a stainless steel covered thrmocouple connected to a digital thermometer is required.The end of glass solvent inlet tube is modified to a fine point, so that the solvent is introdused into the flask as a fine spray.

Soniction:-

This is the method in which Multi lamellar vesicles are transformed to the small unit lamellar vesicles. The ultrasonic irradiation is provided to the MLVs to get the SUVs. There are two methods used. a) Probe sonication method, b) Bath sonication method. The probe is employed for dispersion, which requires high energy in a 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 centrifugation prior to use. Due to above reason most widely the bolt connectors are used Sonication of MLVs is accomplished by placing dispersion into the bath sonicator or placing the tip of the probe sonicator into the test tube of dispersion. (5-10 min.) After sonication applied the resulting 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 the composition and concentration, temperature, sonication, volume and sonication tuning.[8]

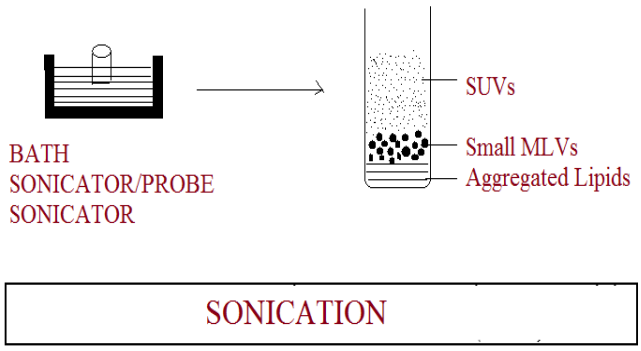

Figure 8: Method of preparation of Liposomes by sonication

Frech pressure cell:-

This method is having the mechanism of high pressure. This method will give the either uni- or oligo- lamellar Liposomes of intermediate size (30-80nm), these Liposomes are more stable compared to the sonicated Liposomes. This method is having some drawbacks are that initial high cost for the press and the pressure cell Liposomes prepared by

this method having less structural defects unlike sonicated liposome.

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 collide together at the right angles at a very high velocity, thereby affecting an efficient transfer of energy. The lipids can be introduced into the fluidizer, either as large MLVs or as the slurry of a hydrated lipid in organic medium. The fluid collected can be recycled through the pump and the interaction chamber until vesicles of spherical dimensions are obtained. DiameterAfter a single pass, the size of vesicles is reduced to a size 0.1 and 0.2um.

Membrane Extrusion technique: - The technique can be used to process LUVs as well as MLVs. The size of Liposomes is reduced by gently passing them through a membrane filter of defined pore size achieved at much lower pressure (<100psi). In this process, the vesicle contents are exchanged with the dispersion medium during the breaking and resealing of phospholipids players as they pass through the polycarbonate membrane. The Liposomes produced by this technique have been termed LUVETs. This technique is most widely used method for an SUV and LUV production for in vitro and in vivo studies.[7]

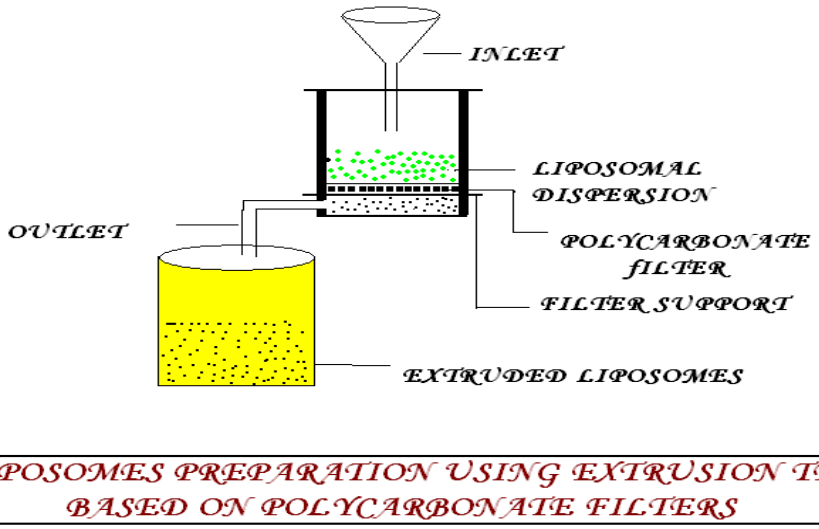

Figure 9: Method of preparation of liposomeby micro emulsification Liposomes

Dried Reconstituted vesicles (DRVs):-

This method starts to freeze drying of a dispersion of empty SUVs. After freeze drying the freeze dried membrane is obtained. Then these freeze dried SUVs are rehydrated with the use of aqueous fluid containing the material to be entrapped. This leads to the formation of the solutes in uni- or oligo- lamellar vesicles.[7]

Freeze Thaw Sonication: - This method is based upon freezing of 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 vesicle formation which are useful for study of membrane transport phenomenon.

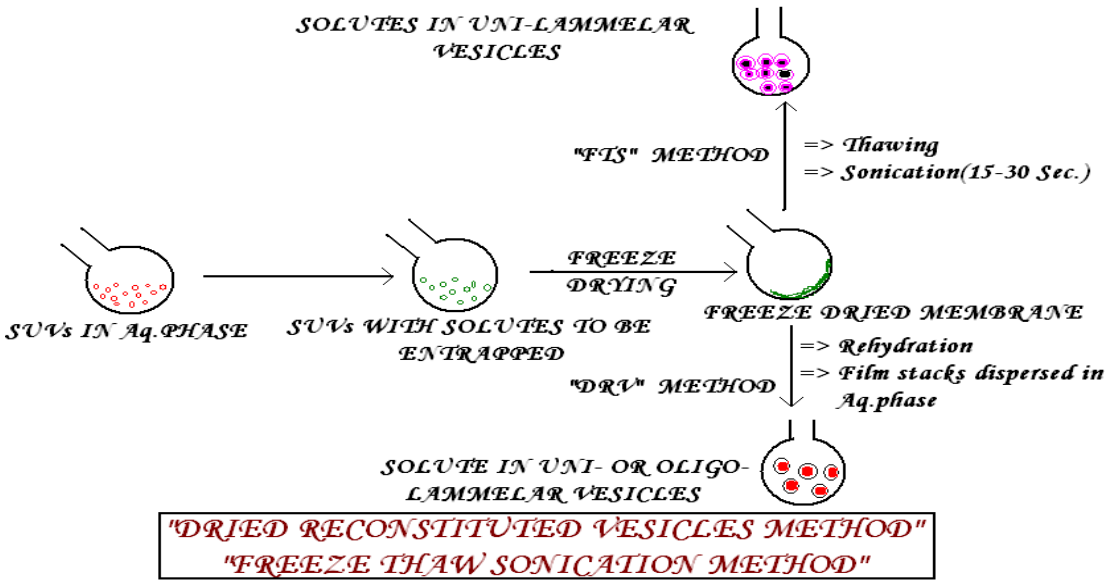

Figure 10: Method of preparation of Liposomes by Freeze Thaw Sonication

pH induced vesiculation

This method prepaer ULVs from MLVs without sonication or high pressure application, they are reassembled by simply charging the pH. It is an electrostatic phenomenon, the transfering chage in ph bring about an increase in the surface charge density of lipid bilayer, provided this exceeeds a certain threshold valve of around 1-2uc/cm2, spontanous vesiculation will occur .the period of exposure of the phospholipid to high ph is less than 2 mins and not long enough to cause detectable

degratation of phospholipd.In this method, dry film of lipid is obtained is the round bottom flask usin the rotatory evaporator and last traces of the solvent are removed using freeze dryer.Then,the film is hydrated with minimum quantity of water by hand shaking at room temperature. At this stage material to be entrapped inside the vesicles may be added in the water before adddition to the lipid.The dispersion is completed by subjecting the suspention to six freeze thawing cycle between 15 degree cesilsius and 5 degrees celsius. The ph of the despersion will be 2.5-3 Sodium hydroxide solution (1m) is added rapidly with mmixing into the suspension then then the ph is redused by addition of 0.1m hcl until a valve of ph 7.5 is achieved.

Calcium induced fusion –

It uses the concept of aggregation and fusion of acid phospholipid vesicles in the presence of calcium .In this method, lipid is dried down and suspended in sonication buffer (Nacl 0.385g, histidin 31.0 mg, this –base 24.2 mg, water 100ml, PH 7.4). The large Liposomes and lipid particles are removed by centrifugation at 100,000g. The Equimolar proportion of calcium solution precipitate is formed. It is incubated for 60 mins at 37 degree Celsius and the precipitate is separated by spinning the container at 3000g for 2o min at room temperature. The supplementing is discarded. The pellet is resuspended is buffered saline containing the material to be entrapped and incubated at 37 degrees Celsius for 10 min. The EDTA (170mm) is added in buffer with mixing The cloudy precipitate will clear rapidly, then incubate for 15 min at 37 degrees Celsius and further 15 min mix at room temperature. Finally, the CA/EDTA complex is removed by dialysis again a letter of buffer.

Solvent Dispersion Techniques

1**) 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 (Hamilton and Guo, 1984).[9] 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, and 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.

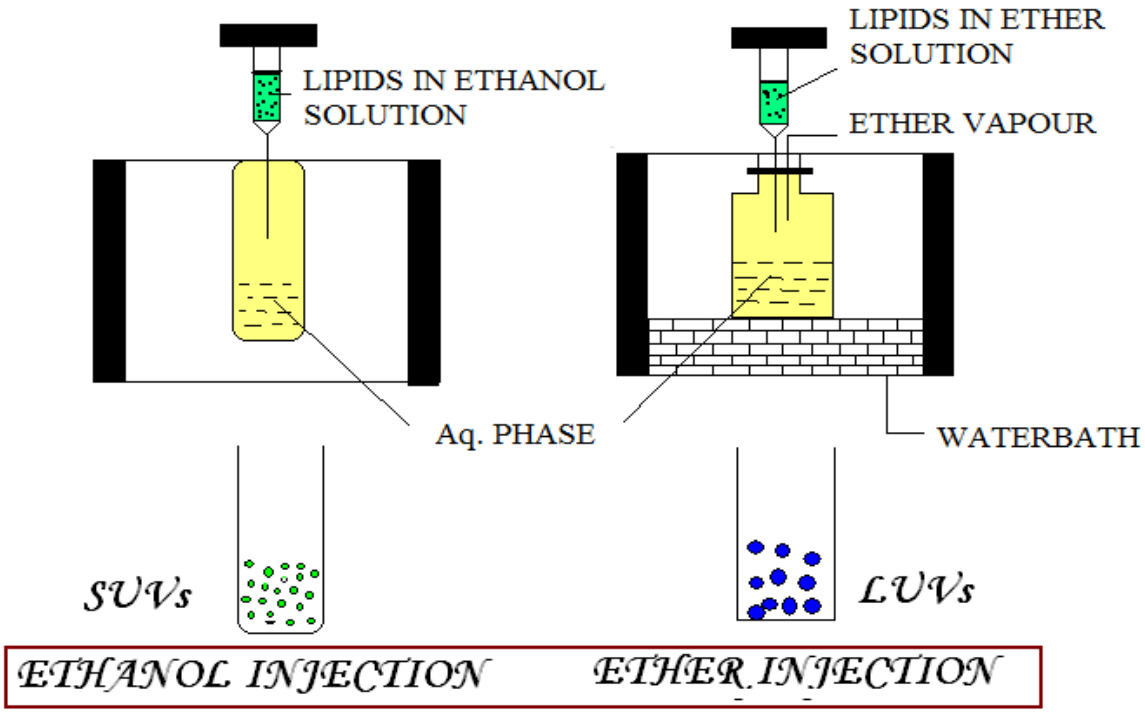

Figure 11: Method of preparation of Liposomes by solvent dispersion

Double Emulsion Vesicles:-

In this method, the outer half of the liposome membrane is created at second intervals between two phases by emulsification of an organic solution in water. If the organic solution, which already contains water droplet, is introduced into an excess aqueous medium followed by mechanical dispersion, multi compartment vesicles are obtained. The ordered dispersion so obtained is described as W/O/W system. If the organic solution, which already contains water droplet, is introduced into an excess aqueous medium followed by mechanical dispersion, multi compartment vesicles are obtained. The ordered dispersion so obtained is described as W/O/W system. At this step monolayers of phospholipids surrounding each water compartment are closely opposed by each other. The next step is to bring about the collapse of a certain proportion of the water droplets by vigorous shaking by using a mechanical vortex mixer. Then the lipid monolayer which enclosed the collapsed vesicle is contributed to adjacent intact vesicle to form the outer leaflet of player of large unilamellar Liposomes. The vesicles formed are unilamellar and are having a diameter of 0.5 micrometers. The encapsulation is found to be 50%.[7]

Reverse phase evaporation vesicles

Sonicated methods (stable plurilamellar vesicles – SPVs) in this method, w/o dispersion is prepared as described earlier with excess lipid, but drying process is accompanied by continued bath sonication with a stream of nitrogen. The redistribution and equilibration of aqueous solvent and solute occur this time in between the various players in each plurilamellar vesicles. The internal structure of SPVs is different from that of MLV-REV, in that they lack a large aqueous medium being located in compartment in between adjacent lamellae. The percent entrapment is normally 30%.

Detergent Removal Methods

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 is brought into intimate contact with the aqueous phase via the intermediary of detergent, which associated with phospholipid molecules from water. In structural formed as a result of this associated are known as missiles, 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 molecules from is known as critical micelle concentration (CMC).[7]

4. Characterization

Table 1: 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	Zeta potential 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

Table 2: 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 3: Biological characterization

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

Physical Characterization

Size and its distribution

Most precise method to determine size of the liposome is by electron microscopy, since it allows to view each individual liposome and to obtain exact information about the profile of liposome population over the whole range of sizes. Unfortunately it is very time consuming and requires equipments that may not always be immediately available to hand. In contrast, laser light scattering (quasi-elastic laser light scattering) method is very simple and rapid to perform but having disadvantage of measuring an average property of the bulk of the Liposomes.

Microscopic methods

Light microscopy has been utilized to examine the gross size/distribution of large vesicles produced

from single chain amphiphiles. If the players are having fluorescent hydrophilic probes, the Liposomes can be examined under a fluorescent microscope. The resolution of the light microscope limits this technique for obtaining the complete size distribution of the preparation. But using negative stain electron microscopy, one can obtain an estimate of the lower end of the size distribution. For the large vesicles (5um), negative stain electron microscopy is not suitable for determination of the size distribution because vesicle distortion during preparation of the specimen makes it difficult to obtain an estimate of the diameter of the original particle. A technical difficulty in obtaining good negative stains of Liposomes is the spreading of the vesicles on the carbon-coated grid.

a) Gel Permeation

Exclusion chromatography on large pure gels was introduced to separate SUVs from radial MLVs. However, large vesicles of 1-3 um diameter usually fails to enter the gel and is retained on the top of the column. A thin layer chromatography system using a garose beads has been introduced as a convenient, fast technique for obtaining a rough estimation of the size distribution of a liposome preparation. However, it was not reported if this procedure was sensitive to a physical blockage of the pores of the agarose gel as is the more conventional column chromatography.

b) Surface Charge

A method using free flow electrophoresis is used to determine the surface charge of MLVs, A technique has been developed that separates extruded vesicles on the basis of their surface charge by electrophoresis on a cellulose acetate plate in a sodium borate buffer pH 8.8. The lipid samples (5 moles) are applied to the plate and electrophoresis is carried out at 4 degrees Celsius on a flat bed apparatus for 30 mins at 18V/cm. The plate is dried and the phospholipids are visualized by the molybdenum blue reagent. Liposomes upto 0. 2 um in diameter can migrate on this sport and with this technique as little as 2 mole % of charge lipids can be detected in a liposome player. This sensitive assay should prove valuable for examining the charge heterogeneity in liposome preparation for following fusion between two populations of vesicles with different charge and for determining.

c) Percent capture (entrapment) It is essential to measure the quantity of material entrapped in Liposomes before the study of the behavior of this entrapped material in physical and biological systems, since the effects observed experimentally will usually be dose related. After removal of unincorporated material by the separation techniques, one may assume that the quantity of material remaining is 100% entrapped, but the preparation may change upon storage. For long term stability test and for developing new liposome

formulations or method of preparation, a technique is needed for separating free of entrapped material.

Mini Column Centrifugation method

In this method, the hydrated gel (sephadexG-50) is filled in a barrel of 1mL syringe without plunger which is plugged with a whatman GF/B filter pad. This barrel is resting in a centrifuge tube. This tube is spun at2000rpm for 3 mins to remove excess saline solution from the gel. After centrifugation the gel column should be dried and have come away from the side of the breathing, eluted saline is removed from the collection tube. Liposome suspension (0.2ml undiluted) is applied dropwise to the top of the gel bed, and the column is spinning at 2000 RPM for 3 min. The Eliot is then removed and set aside for assay.

d) Entrapped Volume The entrapped volume of a population of Liposomes (inuL/mg phospholipid) can often be deduced from measurements of the total quantity of solute entrapped in Liposomes assuring that the concentration of solute in the aqueous medium inside Liposomes is the same as that in the solution used to start with, and assuming that no solute has leaked out of the Liposomes after separation from unentrapped material. However, in many cases such assumption is invalid. For e.g. in two phase methods of preparation, water can be lost from the internal compartment during the drying downstep to remove organic solvent.

e) Lamellarity

The average number of bilayers present in a liposome can be found by freeze electron microscopy and by 31P-NMR. In the latter technique, the signals are recorded before and after the addition of broadening agent such as manganese ions which interact with the outer leaflet of the outermost bilayers. Thus, a 50% reduction in NMR signal means that the liposome preparation is unilamellarand a 25%reduction in the intensity of the original NMR signal means that there are 2 players in the liposome.

Drug Release

The mechanism of drug release from the Liposomes can be assessed by the use of a well calibrated in vitro diffusion cell. The liposome based formulations can be assisted by employing in vitro assays to predict pharmacokinetics andbioavailability of the drug before employing costly andtime-consuming in vivo studies. The dilution-induced drug release in the buffer and plasma was employed as a predictor for the pharmacokinetics performance of liposomal formulations and another assay which determined intracellular drug release induced byLiposomes degradation in the presence of the mouse-liver lysosome list was used to assess the bioavailability of the drug.[7]

Chemical characterization

a) Quantitative Determination of Phospholipids

It is difficult to measure directly the phospholipid concentration, since dried lipids can often contain considerable quantities of residual solvent. Consequently the method most widely used for determination of phospholipidis an indirect one in which the phosphate content of the sample is first measured phospholipids are measured either using Bartlett assay or Stewart Assay.

Bartlett Assay

In the Bartlett assay the phospholipid, phosphorous in the sample is first hydrolyzed to inorganic phosphate. This is converted to phospho-molybdicacid by the addition of ammonium molybdateand phospho-molybdic acid is quantitatively to a blue colour compound by amino-naphthyl-sulfonicacid. The intensity of the blue color is measured spectrophotometrically and is compared with the curve of standards to give phosphorous and hence phosphorlipid content Bartlett assay is very sensitive but is not reasonably reproducible. The problem is that the test is easily upset by tracing contamination with inorganic phosphate.

Stewart Assay

In Stewart assay, the phospholipid forms a complex with ammonium ferrothiocyanate in organic solution. The advantage of this method is that the presence of inorganic phosphate does not interfere with the assay.

b) Phospholipid Oxidation

Oxidation of the fatty acids of phospholipids in the absenceof specific oxidants occurs via a free radical chainmechanism.The initiation step is abstraction of a hydrogen atom from thelipid chain that can occur most commonly as a result of exposure to electro-magnetic radiation or trace amount of contamination with the transition metal ions. Polychain-saturated lipids are particularly prone toxidative degradation. A number of techniques are available for determining theoxidation of phospholipids at different stages i.e., UV absorbance method, TBA method (for endoperoxides), iodometric method (for hydroperoxides) and GLC method.

c) Cholesterol Analysis

Cholesterol is qualitatively analyzed using capillary column of flexible fused silica

Where, as it is quantitatively estimated (in the range of 0-8ug) by measuring the absorbance of purple complex produced with iron upon reaction with a combined reagent containing ferric perchlorate, ethyl acetate and sulfuric acid at 610nm.[10]

Table 4: Application

Application	Utilized
Liposomes in bioengineering	Modern genetic engineering and gene recombinant technology is based on the delivery of genetic material, i.e. fragments of DNA, into various cells and microorganisms in order to alter their genetic code and force them to produce particular proteins or Polypeptides
Medical applications of stealth Liposomes	Sterically stabilized vesicles can act either as long circulating microreservoirs or Tumor (or site of inflammation and infection) targeting vehicles. The former application requires larger Liposomes (0.2 m) while the latter one is due to the ability of small vesicles to leave the blood circulation
Liposomes in anticancer therapy	These cells are in tumours, but also in the gastrointestinal mucosa, hair, and blood cells and therefore this class of drugs are very toxic. The most used and studied is Adriamycin (the commercial name for Doxorubicin HCl).
Macrophage activation and vaccination	The automatic targeting of Liposomes to macrophages can be exploited in several other 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
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

Table 5: List of Marketed Products [8]

Marketed product	Drug used	Target diseases	Company
DoxilTM or CaelyxTM	Doxorubicin	Kaposi's sarcoma	SEQUUS, USA
DaunoXomeTM	DaunSolid tumoursorubicin	Kaposi's sarcoma, breast & lung cancer	NeXstar, USA
AmphotecTM	Amphotericin-B	fungal infections, Leishmaniasis	SEQUUS, USA
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
EvacetTM	Doxorubicin	Metastatic breast cancer	The liposome company, USA

Table 6: Some Liposomal Cosmetic Formulations Currently on the Market[11]

Product	**Manufacturer**	**Liposomes and key ingredients**
Efect du Soleil	L'Or´eal	Tanning agents in Liposomes Niosomes Lancome
Niosomes	Lancome (L'Or´eal)	Glyceropolyether with moisturizers
Nactosomes	Lancome (L'Or´eal	Vitamins
Formule Liposome Gel	Payot (Ferdinand Muehlens)	Thymoxin, hyaluronic acid
Future Perfect Skin Gel	Estee Lauder	TMF, vitamins E, A palmitate, cerebroside ceramide.
Symphatic 2000	Biopharm GmbH	Thymus extract, vitamin A palmitate
Natipide II	Nattermann PL	Liposomal gel for do-it-yourself
Flawless finish	Elizabeth Arden	Liquid make-up
Inovita	Pharm/Apotheke	Thymus extract, hyaluronic
Eye Perfector	Avon	Soothing cream to reduce eye

CONCLUSION

Liposomes over the years have been investigated as the major drug delivery systems due to their flexibility to be tailored for varied desirable purposes. The flexibility in their behaviour can be exploited for the drug delivery through any route of administration and for any drug or material irrespective of its physicochemical properties. The uses of Liposomes in the delivery of drugs and genes to tumour sites are promising and may serve as a handle for focus of future research. Liposomes are best tool for targeting brain; hence it is made up of lipid bilayer. And also it's having a site specific delivery and it produces long term therapy. BBB is made up of lipid bilayer so when compared to other dosage form Liposomes are easily crosses the B.B.B. Hence we can achieve very good site specific action via Liposomes.

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