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LIPOSOMES: FROM CONCEPT TO COMMERCIALIZATION

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ABSTRACT
Liposomes are defined as phospholipid vesicles formed spontaneously by dispersion of lipid films in an aqueous environment to form particles with an aqueous interior surrounded by one or more concentric bilayers of phospholipids with a diameter ranging from ~30 nm to several microns. Liposomes can be composed of naturally-derived phospholipids with mixed lipid chains or surfactants. Although liposome formation is actually a spontaneous process, the current trend is to classify them into a class of pharmaceutical devices in the nanoscale range engineered by physical and/or chemical means and referred to as nanomedicines. Liposomes can be filled with drugs and used to deliver drugs for cancer and other diseases. Nanomedicines are a direct result of the application of nanotechnology to medicine and encompass molecular and supramolecular devices such as liposomes and other nanoparticulate carriers. Amongst the various carriers, few drug carriers reached the stages of clinical trials where phospholipid vesicles (liposome) show strong potential for effective drug delivery to the site of action. Strategies used to enhance liposome-mediated drug delivery *in vivo* include the enhancement of stability and circulation time in the bloodstream, targeting to specific tissues or cells and facilitation of intracytoplasmic delivery. As drug carriers, liposomes have great potential in that selective targeting and release rate control of drugs can be performed by appropriate modifications to the carrier itself, without altering the structure of original drugs. Selective targeting of liposomes to specific tissues such as hepatocytes, macrophages and tumors has been performed in vitro as well as in vivo.

KEYWORDS: Liposomes, Phospholipids, Extrusion, High pressure homogenization.

INTRODUCTION
Liposome was discovered about 40 years ago by Bangham and co-workers and was defined as microscopic spherical vesicles that are formed when phospholipids are hydrated or exposed to an aqueous environment. Liposomes are spherical, self-closed vesicles of colloidal dimensions, in which (phospho) lipid bilayer sequesters part of the solvent, in which they freely float, into their interior.[1] The typical characteristic of bilayer forming lipids is their amphiphilic nature: a[1] polar head group covalently attached to one or two hydrophobic hydrocarbon tails. 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. In the case of one bilayer encapsulating the aqueous core one speaks either of small or large unilamellar vesicles while in the case of many concentric bilayers one defines large multilamellar vesicles.[2]

They are classified structurally into multilamellar vesicles (MLVs) and unilamellar vesicles (ULVs). ULVs have a single phospholipid bilayer membrane and a diameter of 0.05–0.25 µm. These liposomes (i.e., ULVs) can be further classified into large unilamellar vesicles (LUVs) with a diameter of 0.05– 0.25 µm and small unilamellar vesicles (SUVs) with a diameter of 0.05–0.10µm. In contrast to lipid monolayer structures, liposomes are characterized by extended, two-dimensional and clearly separated hydrophilic and hydrophobic regions. The hydrophilic portions of bilayer lipids are directed towards aqueous phases (external and internal), whereas hydrophobic portions of both lipid layers are directed towards one another, forming the internal core of a membrane. Liposome can carry drugs in one or three potential compartments (water soluble agents in the central aqueous core, lipid soluble agents in the membrane, peptide and small proteins at the lipid aqueous interface). A special characteristic of liposomes for drug delivery is that they enable water-soluble and water-insoluble materials to be encapsulated together. Water-soluble materials are entrapped in the aqueous

core, while water-insoluble and oil soluble hydrophobic drugs reside within the lipid bilayer. Due to their structure, chemical composition and colloidal size, all of which can be well controlled by preparation methods, liposomes exhibit several properties which may be useful in various applications. The most important properties are colloidal size, i.e. rather uniform particle size distributions in the range from 20 nm to 10 µm and special membrane and surface characteristics. They include bilayer phase behavior, its mechanical properties and permeability, charge density, presence of surface bound or grafted polymers, or attachment of special ligands, respectively. Additionally, due to their amphiphilic character, liposomes are a powerful solubilizing system for a wide range of compounds. In addition to these physico-chemical properties, liposomes exhibit many special biological characteristics, including (specific) interactions with biological membranes and various cells.[3] These properties point to several possible applications with liposomes as the solubilizers for difficult-to-dissolve substances, dispersants, sustained release systems, delivery systems for the encapsulated substances, stabilizers, protective agents, microencapsulation systems and microreactors being the most obvious ones. Liposomes can be made entirely from naturally occurring substances and are therefore nontoxic, biodegradable and non immunogenic. In addition to these applications which had significant impact in several industries, the properties of liposomes offer a very useful model system in many fundamental studies from topology, membrane biophysics, photophysics and photochemistry, colloid interactions, cell function, signal transduction, and many others.[3-5] Due to their high degree of biocompatibility, liposomes were initially conceived of as systems for intravenous delivery. It has since become apparent that liposomes can also be useful for delivery of drugs by other routes of administration. The formulator can use strategies to design liposomes for specific purposes, thereby improving the therapeutic index of a drug by increasing the percent of drug molecules that reach the target tissue, or, alternatively, decreasing the percent of drug molecules that reach sites of toxicity. The industrial applications include liposomes as drug delivery vehicles in medicine, adjuvants in vaccination, signal enhancers/carriers in medical diagnostics and analytical biochemistry, solubilizers for various ingredients as well as support matrix for various ingredients and penetration enhancer in cosmetics. Liposomes have been studied for many years as carrier systems for drugs 10 with advantages such as enhancement of therapeutic efficacy at low dosage and, hence, reduction in toxicity of the encapsulated agent; improved pharmacokinetic profiles, e.g., enhanced tissue penetration and increased biological half life; targeting to tumour tissues, e.g., liposomal doxorubicin; and increased stability of the drug particularly against enzymatic degradation.[6-8] Although there are approximately 15,000 publications dealing with liposomes, very few are centered on the pharmaceutical issues that must be addressed to bring liposomal products to the marketplace.

Structural Components of Liposome

There are number of the structural and nonstructural components of liposomes, major structural components of liposomes are:

a. Phospholipids

Phospholipids are the major structural component of biological membranes, where two type of phospholipids exist- PHOSPHODIGLYCERIDES AND SPHINGOLIPIDS. The most common phospholipid is phosphatidylcholine (PC) molecule. Molecule of phosphatidylcholine are not soluble in water and in aqueous media they align themselves closely in plannar bilayer sheets in order to minimize the unfavorable action between the bulk aqueous phase and long hydrocarbon fatty chain. 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 Examples of phospholipids are:

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

b. Cholesterol

Cholesterol dose not by itself form bilayer structure but can be incorporated into phospholipid membranes in very high concentration upto 1:1 or even 2:1 molar concentration of cholesterol to phosphatidylcholine. Cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface and aliphatic chain aligent parallel to the acyl chains in the center of the bilayer. The high solubility of cholesterol in phospholipid liposome has been attributed to both hydrophobic and specific headgroup interation, but there is no unequivocal evidence for the arrangement of cholesterol in the bilayer.[9-10]

Phase Behavior of Liposomes

An important feature of membrane lipids is the existence of a temperature-dependent reversible phase transition, where the hydrocarbon chains of the phospholipid undergo a transformation from an ordered (gel) state to a more disordered fluid (liquid crystalline) state. These changes have been documented by freeze-fracture electron microscopy but are most easily demonstrated by differential scanning calorimetry. The physical state of the bilayer profoundly affects the permeability, leakage rates and overall stability of the liposomes. The phase transition temperature Tm is a function of the phospholipid content of the bilayer (Table 1). By proper admixture of bilayer-forming materials, one may design liposomes to "melt" at any reasonable temperature. This strategy has been used to deliver methotrexate to solid

tumors, which are heated to the phase transition temperature of the custom-designed liposomal phospholipids. The phase transition temperature can be altered by using phospholipid mixtures or by adding sterols such as cholesterol. The Tm-value can give important clues as to liposomal stability and permeability and as to whether a drug is entrapped in the bilayer or the aqueous compartment.

Table 1 Phase Transition Temperatures of Some Synthetic Phospholipids Used to Prepare Liposomes

Lipid Charge	T_m	(°C)
Dilauroyl phosphatidylcholine	0	0
Dimyristoyl phosphatidylcholine	0	23
Dipalmitoyl phosphatidylcholine	0	41
Dimyristoyl phosphatidylethanolamine	0	48
Distearoyl phosphatidylcholine	0	58
Dipalmitoyl phosphatidylethanolamine	0	60
Dioleoyl phosphatidylglycerol	-1	-18
Dilauroyl phosphatidylglycerol	-1	4
Dimyristoyl phosphatidylglycerol	-1	23
Dipalmitoyl phosphatidylglycerol	-1	41
Distearoyl phosphatidylglycerol	-1	55

Mechanism of Liposome Formation

Phospholipids are amphipathic having affinity for both aqueous and polar moieties molecules as they have a hydrophobic tail and a hydrophilic or polar head. The hydrophobic tail is composed of two fatty acid chain containing 10-24 carbon atom and 0-6 double bonds in each chain. The macroscopic structures most often formed include lamellar, hexagonal or cubic phases dispersed as colloidal nanoconstructs (artificial membranes) referred to as liposomes, 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.[11]

Application of Double Layer Theory to Liposomes

Once assembled, liposomes behave in much the same way as other charged colloidal particles suspended in water or electrolyte solution. Under conditions where the charge on each particle is weak, the electrostatic repulsive force among the particles is also weak, increasing the opportunity for close approach. Some neutral particles tend either to flocculate or aggregate and sediment from suspension for this reason. Similarly, two populations of liposomes bearing opposite electric charges will aggregate at a rate that is a function of the electrostatic attractive forces among the particles. Particles bearing net negative charges may be induced to aggregate strongly in the presence of di- or trivalent cations. For example, calcium in the 1–2 mM range will induce liposomes containing more than 50 mol% PS to aggregate. These phenomena have dramatic effects on the physical stability of liposomes and lead to fusion of liposomes with one another resulting in increases in their overall size; like aggregation, particle size growth, particularly during storage, would be undesirable in most products. Fortunately the tendency of liposomes to aggregate and fuse can be controlled by the inclusion of small amounts of negatively charged lipids such as PS or PG or positively charged amphiphiles such as stearylamine in the formulation. Knowing the number and the sign of charged groups added and the valency and concentration of electrolytes in the medium, the magnitude of the electrostatic forces generated by these charged groups can be closely approximated by using the double layer theory. These results can then be correlated with physical stability of liposomes and used to guide formulation efforts. The amount of charged component and ionic conditions in a particular liposome dosage form can be adjusted to produce a high-enough zeta potential to inhibit close approach of vesicles and prevent their aggregation. In practice it is usually necessary to determine empirically the magnitude of the zeta potential required to prevent aggregation in a particular system. However, once this has been done, it is possible to use the zeta potential as a quality control check to insure that each batch of liposomes contains sufficient charged groups to avoid aggregation during storage.

Advantages of Liposome

Provides selective passive targeting to tumour tissue (liposomal doxorubicin).

1. Liposome increase 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 nonimmunogenic for systemic and non-systemic administrations.
4. Liposomes reduce the toxicity of the encapsulated agent (Amphotericin B, Taxol).
5. Liposomes help to reduce exposure of sensitive tissues to toxic drugs.
6. Site avoidance effect.
7. Flexibility to couple with site-specific ligands to achieve active targeting.

Disadvantages of Liposome

1. Production cost is high.
2. Leakage and fusion of encapsulated drug / molecules.
3. Sometimes phospholipid undergoes oxidation and hydrolysis like reaction.
4. Short half-life.
5. Low solubility.
6. Fewer stables.

A. CHARACTERIZATION OF LIPOSOMES[12-14]

Liposome prepared by one of the preceding method must be characterized. The most important parameters of liposome characterization include visual appearance, turbidity, size distribution, lamellarity, concentration, composition, presence of degradation products and stability.

1. Visual Appearance

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

2. Determination of Liposomal Size Distribution

Size distribution is normally measured by dynamic light scattering. This method is reliable for liposomes with relatively homogeneous size distribution. A simple but powerful method is gel exclusion chromatography, in which a truly hydrodynamic radius can be detected. Sephacryl-S100 can separate liposome in size range of 30-300nm. The average size and size distribution of liposomes are important parameters with respect to physical properties and biological fate of the liposomes and their entrapped substances. There are a number of methods used to determine this parameter, but the most commonly used methods are the following.

a. Light Scattering

A variety of techniques are available to size liposomes based on light scattering. The popularity of this method depends on its ease of operation and the speed by which one can obtain data. The newer instruments are based on dynamic laser light scattering. If the liposomes to be analyzed were monodisperse, light scattering would be the method of choice. Unfortunately, most preparations are heterogeneous, and they require an accurate estimation of their size-frequency distributions. Light scattering methods rely on algorithms to determine particle size distribution and the results obtained can be very misleading. Some complex algorithms have been developed in an attempt to deal with this problem. Furthermore, such methods cannot distinguish between a large particle and a flocculated mass of smaller particles.

b. Light Microscopy

This method can be used to examine the gross size distribution of large vesicle preparations such as MLVs. The inclusion of a fluorescent probe in the bilayer permits examination of liposomes under a fluorescent microscope and is a very convenient method to obtain an estimate of at least the upper end of the size distribution.

c. Negative Stain Electron Microscopy

This method, using either molybdate or phosphotungstate as a stain, is the method of choice for size distribution analysis of any size below 5 mm. It should be used to validate light scattering data that will ultimately be used for quality assurance. For accurate statistical evaluation (±5%), one should count at least 400 particles and not rely on a single specimen for counting.

d. Freeze Fracture Electron Microscopy

This method is especially useful for observing the morphological structure of liposomes. Since the fracture plane passes through vesicles that are randomly positioned in the frozen section, resulting in non mid-plane fractures, the observed profile diameter depends on the distance of the vesicle center from the plane of the fracture. Mathematical methods have been devised to correct for this effect.

e. Cryoelectron Microscopy

This is a relatively new technique that allows direct observation of quickly frozen samples without any staining and is, therefore, the least prone to artifacts. Numerous tests have shown that very quick freezing can preserve the structure, while it may give rise to unreal size distribution due to the fact that larger particles are excluded from the thin (0.2–0.4 mm) film of ice on the microscopic grid.[5]

f. Gel Chromatography

Since the introduction of large pore size gel (Sephacryl S 1000), an easy and quantitative determination of liposome size distribution is possible. In contrast to all other techniques, this method gives a true (i.e., "fit-independent") distribution according to their true hydrodynamic radius for liposomes smaller than 0.3–0.4 mm.[5]

3. Determination of Lamellarity

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, The average number of bilayers present in liposomes can be found by freeze-fracture electron microscopy and 31P-NMR. In the latter technique, the signals are recorded before and after the addition of non-permeable broadening agent such as Mn^{2+}. Manganese ions interact with the outer leaflet of the outermost bilayer. Thus, a 50% reduction in NMR signal means that the liposome preparation is unilamellar and a 25% reduction in the intensity of the original NMR signal means there are two bilayers in the liposomes.[15]

4. 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.

5. Surface Charge

Liposomes 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.

6. Drug entrapment

1. Factors Affecting Drug Entrapment

The amount and location of a drug within a liposome is dependent on a number of factors. The location of drug within a liposome is based on the partition coefficient of the drug between aqueous compartments and lipid bilayers and the maximum amount of drug that can be entrapped within a liposome is dependent on its total solubility in each phase. The total amount of liposomal lipid used and the internal volume of the liposome will affect the total amount of nonpolar and polar drug, respectively, that can be loaded into a liposome. Efficient capture will depend on the use of drugs at concentrations that do not exceed the saturation limit of the drug in the aqueous compartment (for polar drugs) or the lipid bilayers (for nonpolar drugs). The method of preparation can also affect drug location and overall trapping efficiency. Incorporation of drugs that have intermediate partition coefficients (significant solubility in both the aqueous phase and the bilayer) may be undesirable. If liposomes are prepared by mixing such a drug with the lipids, the drug will eventually partition to an extent depending on the partition coefficient of the drug and the phase volume ratio of water to bilayer. Also, the rate of partitioning will be a function of its diffusivity in each phase. Release rates (a measure of instability) are highest when the drug has an intermediate partition coefficient. Bilayer/aqueous compartment partition coefficients are usually estimated by determining their organic solvent/water (e.g., octanol/water) partition coefficients. They can also be determined precisely by a method described by Bakouche and Gerlier,[**Error! Reference source not found.**] which is based on the physical separation of the aqueous and bilayer phases by ultracentrifugation after mechanical (ultrasonics at low temperatures) disruption of the liposomes followed by analysis of each phase for drug.

2. Internal Volume and Encapsulation Efficiency

Internal volume and encapsulation efficiency are two parameters used to describe entrapment of water-soluble drugs in the aqueous compartments of liposomes. The internal or trapped or capture volume is expressed as aqueous entrapped volume per unit quantity of lipid (mL/mmol or mL/mg). It is determined by entrapping a water-soluble marker such as 6-carboxyfluorescein, 14C or 3H-glucose or sucrose and then lysing the liposomes by the use of a detergent such as Triton X-100. Determination of the amount of marker that was trapped enables one to back-calculate the volume of entrapped water. The encapsulation efficiency describes the percent of the aqueous phase (and hence the percent of water-soluble drug) that becomes entrapped during liposome preparation. The remaining drug remains outside of the liposome and is therefore "wasted." Encapsulation efficiency is usually expressed as percentage entrapment per milligrams of lipid. The internal or trapped volume and encapsulation efficiency greatly depend on liposomal content, liposomal size, lipid concentration, method of preparation and the drug used. Incorporation of charged lipids into bilayers increases the volume of the aqueous compartments by separating adjacent bilayers due to charge repulsion, resulting in increases in trapped volume. It should be pointed out that for hydrophobic drugs; entrapment efficiency usually approaches 100% almost irrespective of liposomal type and composition. This high encapsulation efficiency, however, is normally observed only in the test tube, while upon application or simple dilution, the majority of the drug is quickly lost from the liposomes. The same is true for water-soluble drugs with good membrane permeability such as antibiotics and many anticancer agents. Some drugs, which are weak acids or weak bases, can be loaded into liposomes by the use of a pH gradient.[16] Recently ammonium salt gradient was introduced,[18] which, in addition, can cause the precipitation of the drug in the liposome interior and thus greatly increase the stability of the encapsulation.[19]

Factors effecting drug entrapment

1. Slow versus Fast Hydration, Thickness of the Lipid Film

The time allowed for hydration and conditions of agitation are critical in determining the amount of the aqueous buffer (or drug solution) entrapped within the internal compartments of the MLV. For example, Szoka and Papahadjopoulos[19], reported that a similar lipid concentration can encapsulate 50% more aqueous buffer per mole of lipid when hydrated for 20 h with gentle shaking, compared to a hydration period of 2 h with vigorous shaking, despite the fact that the two preparations exhibit a roughly similar particle size distribution. If hydration time is reduced to a few minutes with vortexing, a suspension will exhibit a still lower capture volume and a smaller mean diameter. Bangham[20] showed that the hydration and entrapping process is most efficient when the film of dry lipid is kept thin. This means that different-sized round-bottom flasks should be used for different quantities of lipid. Glass beads have been used by some investigators to increase the surface area available for film deposition. Thus the hydration time, method of suspension of the lipids and the thickness of the film can result in markedly different preparations of MLVs, in spite of identical lipid concentrations and compositions and volume of the suspending aqueous phase.

2. Effect of Charged Lipids

The presence of negatively charged lipids, such PS, PA, PI or PG, or positively charged detergents such as stearylamine, will tend to increase the interlamellar distance between successive bilayers in the MLV structure and thus lead to a greater overall entrapped volume. This is particularly true in low ionic strength buffers or nonelectrolytes (such as sucrose), since the electrostatic repulsive forces, which give rise to the effect, are greater under these conditions. Generally, about a 10–20 mol percent of a charged species is used, although it is possible to produce MLVs from a singly charged lipid such as PS. The presence of charged lipids also reduces the likelihood of aggregation following the formation of MLVs.

3. Hydration in the Presence of Solvent

MLVs with high entrapment of solutes can be produced by hydrating the lipid in the presence of organic solvents. A method introduced by Papahadjopoulos and Watkins[21] begins with a two-phase system consisting of equal volumes of petroleum ether containing bilayer-forming lipids and of an aqueous phase. The contents of the tube are emulsified by vigorous vortexing and the ether is removed by passing a stream of nitrogen gas over the mixture. As the ether is removed in the carrier gas, MLVs form in the aqueous phase. A similar method was reported by Gruner et al.[22] except that diethyl ether was used as the solvent, sonication was used in place of vortexing and the aqueous phase was reduced to a relatively small proportion. Typically, the lipids are dissolved in about 5 mL ether and about 0.3 mL of the aqueous phase to be entrapped is added. The two phases are emulsified by sonication while a gentle stream of nitrogen gas is passed over the mixture. The resulting MLV preparation encapsulates up to 40% of the solvent throughout the hydration step and the concentration of solute molecules is in equilibrium across all the bilayers, a feature that is claimed to translate into enhanced stability to leakage.

B. Liposome Preparation Methods

General Methods of Preparation

All the method of preparing liposomes involves four basic stages:

1. Drying down lipids from organic solvent.
2. Dispersion of lipid in aqueous media.
3. Purification of resultant liposome.
4. Analysis of final product.

Method of Liposome Preparation and Drug Loading[23]

The original method of Bangham et al.[24] for the preparation of liposomes involves the deposition of a thin lipid film from an organic solvent medium on the walls of a container, followed by agitation with an aqueous solution of the material to be encapsulated. If the agitation is carried out above the gel-liquid phase transition temperature of the phospholipid and the film is kept thin, multilamellar vesicle (MLVs) form spontaneously. Drug trapping efficiency of these MLVs is low and the method cannot be easily scaled up. Large unilamellar vesicles (LUVs) are produced, but the encapsulating efficiency is not high and the material to be entrapped requires possible detrimental contact with organic solvent. Small unilamellar vesicles (SUVs) are formed by first dispersing a phospholipid and a surfactant in an aqueous solution of the material to be encapsulated. The dispersion of mixed micelles is then exhaustively dialyzed to remove surfactant and a homogeneous dispersion of SUVs is obtained.[25] The degree of encapsulation is low due to loss of material during dialysis, except in the case of macromolecules and significant residual surfactant is present.

Various methods used for the preparation of liposome:-

1. Passive loading techniques

Passive loading techniques include three different methods:

a. Mechanical dispersion method

- **Lipid film hydration by hand shaking, non hand shaking**

Multilamellar vesicles are by far the most widely studied type of liposome and as pointed out by Alec Bangham in 1964, exceptionally simple to make. In general a mixture of lipids is deposited as a thin film on the bottom of a round-bottom flask by rotary evaporation under reduced pressure. MLVs form spontaneously when an excess volume of aqueous buffer is added to the dry lipid. However, in many cases, MLVs have not been rigorously characterized with respect to size, polydispersity, number of lamallae, encapsulated volume and stability. Due to their ease of production, many investigators have simply made a preparation of MLVs for use in both in vitro and in vivo experiments without taking the time to fully characterize them. This has led to a great deal of confusion in the interpretation of experimental results, because, as will be explained below, minor changes in the method of preparation can lead to major differences in the behavior of liposomes. Large unilamellar and oligolamellar vesicles with high entrapment efficiencies have been formed by a clever method reported by Shew and Deamer.[26] In this method, sonicated vesicles are mixed in an aqueous solution with the solute desired to be encapsulated and the mixture is dried under a stream of nitrogen. As the sample is dehydrated, the small vesicles fuse to form a multilamellar film that effectively sandwiches the solute molecules between successive layers. Upon rehydration, large vesicles are produced that have encapsulated a significant proportion of the solute. The optimal mass ratio of lipid to solute was reported to be approximately 1:2 to 1:3. This method has been applied in a number of settings, since it depends only on controlled drying and rehydration processes and does not require extensive use of organic solvents, detergents, or dialysis systems.

- **Freeze drying**

A simple method for preparing MLVs with high entrapment efficiency was developed by Ohsawa et al.[27]

and Kirby and Gregoriadis.[7] The aqueous phase containing the molecules to be encapsulated is mixed with a preformed suspension of SUVs and the mixture is freeze-dried by conventional means. Large MLVs are formed when the dry lipid is rehydrated, usually with a small volume of distilled water. Encapsulation efficiencies up to 40% have been reported for this method.

- **Microfluidization**

A recent technique called microfluidization is based on impinging two fluidized streams at high velocity. The method claims greater uniformity, smaller sizes and high capture rates, up to 75%. A method based on micro-emulsification/homogenization was developed for the preparation of liposomes. MICROFLUIDIZER is available from Microfluidics Corporation, Massachusetts, USA. A plot plant based on this technology can produce about 20 gallon/minute of liposomes in 50-200 nm size range.

- **Sonication**

The preparation of sonicated SUVs has been reviewed in detail by Bangham.[30] Briefly, the usual MLV preparation is subsequently sonicated either with a bath-type sonicator or a probe sonicator under an inert atmosphere (usually nitrogen or argon). Although probe sonication leads to more rapid size reduction of the MLVs, degradation of lipids, metal particle shedding from the probe tip and aerosol generation can present problems. Bath-type sonicators also have disadvantages (such as the need to pay greater attention to position of the tube and water level in the bath), but temperature can be accurately regulated. Also, the tube containing the specimen is sealed allowing for aseptic operations and little likelihood of personnel exposure to aerosols.

- **French pressure cell**

Dispersions of MLVs can be converted to SUVs by passage through a small orifice under high pressure. A French pressure cell was used by Hamilton et al.[30] for this purpose. MLV dispersions are placed in the French press and extruded at about 20,000 psi at 4°C. One pass through the cell produces a heterogeneous population of vesicles ranging from several microns in diameter to SUV size. Multiple extrusions result in a progressive decrease in the mean particle diameter. Following about four to five passes, approximately 95% of the vesicles are converted to SUVs as judged by size exclusion chromatography. The resulting vesicles are somewhat larger than sonicated SUVs ranging in size from 30 to 50 nm. The method is simple, reproducible and nondestructive. However, temperature control is difficult (the pressure cell must be allowed to cool between extrusions or the temperature rise may damage the lipids) and the working volumes are relatively small (about 50 mL maximum).

- **Membrane extrusion**

As mentioned above, MLV suspensions rich in acidic lipids, such as PS or PG, tend to have large interbilayer distances and large internal aqueous cores due to electrostatic repulsive forces among the bilayers. Moreover, as the average size is reduced, the vesicles become more and more single layered. The mechanism at work during such high-pressure extrusion appears to be much like the peeling of an onion. As the MLVs are forced through the small pores, successive layers are "peeled" off until only one remains. For this method to generate truly single-layered vesicles, however, the aqueous core of the starting MLV must be greater than about 70 nm in diameter. Although this appears to be the case for vesicles composed predominantly of acidic lipids, neutral vesicles or vesicles with only a few mole percent acidic lipids are not likely to convert to true single lamellar vesicles using this technique, because the diameter of the inner most bilayer is probably significantly less than 70 nm.

- **Freeze-thawed liposomes**

A method for the reconstitution of membrane proteins based on rapid freezing of sonicated phospholipid mixtures followed by thawing and brief sonication was originally described by Kasahara and Hinkle.[32] Formation of large liposomes by this technique probably results from the fusion of small vesicles during freezing and/or thawing of the suspension of small vesicles. This type of fusion is strongly inhibited by increasing the ionic strength of the medium, e.g., adding sucrose and by increasing the lipid concentration. For an unexplained reason, pure phosphatidylcholine vesicles do not appear to be good candidates for this type of fusion induced growth. The method involves the formation of fully hydrated small vesicles in dilute buffer by sonication followed by freeze/thawing in the presence of high concentrations of the electrolyte of interest in order to induce equilibration of the electrolyte across the bilayer membranes of the small vesicles. In the final step of the process, the electrolyte concentration is reduced by dialysis against dilute buffer. This results in the influx of water into the small vesicles (driven by the osmotic imbalance) causing them to swell and fuse into giant vesicles. The method is rather involved and not easily scaled up.

b. Solvent dispersion method

- **Ether injection**

A method introduced by Deamer and Bangham in 1976[33] provides a means of making SUVs by slowly introducing a solution of lipids dissolved in diethyl ether (or ether/methanol mixtures) into warm water having temperature high enough for the solvent to be rapidly evaporated. Typically, the lipid mixture is injected into an aqueous solution of the material to be encapsulated (using a syringe-type infusion pump) at 55–65°C or under reduced pressure. Subsequent removal of residual ether under vacuum leads to the formation of single-layer vesicles. Depending on the condition used, the diameter of the resulting vesicles ranges from 50–200 nm. The usual lipid concentration is about 2 mg/mL ether and

about 2 mL of this solution is infused into 4 mL of the aqueous phase at a rate of 0.2 mL/min at 50–60°C.

- **Ethanol Injection**

An alternative method for producing SUVs that avoids both sonication and exposure to high pressure is the ethanol-injection technique described by Batzri and Korn.[34] Lipids dissolved in ethanol are rapidly injected into a vast excess of buffer solution forming SUVs spontaneously. The procedure is simple and rapid, and avoids exposure to harsh conditions of both lipids and the material to be entrapped. Unfortunately, the method is restricted to the production of relatively dilute SUV suspensions. The final concentration of ethanol cannot exceed about 10% by volume, or the SUVs will not form. Removal of residual ethanol can also present a problem, since ethanol forms an azeotrope with water, which is difficult to remove under vacuum or by distillation. Various available ultrafiltration apparatus may be used to both concentrate the suspension and remove ethanol, but, these procedures tend to be slow and expensive to scale up. Another limitation of the method is related to the susceptibility of various biologically active macromolecules to inactivation in the presence of even low amounts of ethanol.

- **Reverse phase evaporation vesicles**

The reverse micelle method[35] starts with the dispersion of an aqueous solution of the material to be encapsulated in a volatile organic solvent containing the lipid mixture. This step is followed by redispersing in an aqueous buffer medium to form a W/O/W multiple emulsion. On evaporation of the solvent, SUVs are obtained. The reverse phase evaporation method[**Error! Reference source not found.**] is based on the transformation of an emulsion to a liposomal dispersion. LUVs can also be prepared by forming a water-in-oil emulsion of phospholipids and buffer in excess organic phase followed by removal of the organic phase under reduced pressure (the so called "Reverse Phase Evaporation," or REV, method). The two phases are usually emulsified by sonication, but other mechanical means have also been used. Removal of the organic solvent under vacuum causes the phospholipid-coated droplets of water to coalesce and eventually form a viscous gel. Removal of the final traces of solvent results in the collapse of the gel into a smooth suspension of LUVs. The method, which was pioneered by Szoka and Papahadjopoulos in 1978[36], has been used extensively for applications that require high encapsulation of a water-soluble drug. Entrapment efficiencies up to 65% can be obtained with this method. The phospholipids are first dissolved in an organic solvent such as ethylether, isopropylether or mixtures of two solvents, such as isopropylether and chloroform. The emulsification is most easily accomplished when the density of the organic phase matches that of the buffer (i.e., about 1). For this reason, ether (density of about 0.7) is often mixed with a solvent of higher density such as trichlorotrifluoroethane (density of 1.4), to produce a solvent system with a density close to water. The aqueous phase containing the material to be entrapped is added directly to the phospholipid-solvent mixture. The ratio of aqueous phase to organic phase is usually about 1:3 for ether and 1:6 for isopropylether-chloroform mixtures. Preparations using even greater proportions of organic phase have been reported. The two phases are emulsified by sonication for a few minutes and the organic phase is removed slowly under a partial vacuum produced by a water aspirator on a rotary evaporator at 20–30°C. The vacuum is usually maintained at about 500 microns for the first few minutes (using a nitrogen gas bleed to lower the vacuum and a gauge to measure the vacuum) and then raised cautiously to fill the aspirator vacuum to prevent the ether from evaporating too quickly.

c. Detergent removal method

- **Detergent (cholate, alkylglycoside, Triton X- 100) removal form mixed micelles**

An essentially different approach to produce liposomes is dependent on the removal of detergent molecules from aqueous dispersions of phospholipid/detergent mixed micelles. As the detergent is removed, the micelles become progressively richer in phospholipid and finally coalesce to form closed single-bilayer vesicles. Three methods of detergent removal appropriate for this purpose have been described in the literature and are treated separately below.

i.Dialysis

Kagawa and Racker[37] were the first to introduce the dialysis method for lipid vesicle preparation. Although these authors were primarily interested in reconstituting biological membranes solubilized with detergents, their method is applicable to the formation of liposomes as well. Detergents commonly used for this purpose exhibit a reasonably high critical micelle concentration (on the order of 10–20 mM) in order to facilitate their removal and include the bile salts sodium cholate and sodium deoxycholate and synthetic detergents such as octylglucoside. The treatment of egg PC with a 2:1 molar ratio of sodium cholate followed by dialysis results in the formation of vesicles in the 100 nm diameter range within a few hours. Another modification of the cholate removal technique is one in which the rate of efflux of the detergent from the mixture is controlled. This procedure, described in detail by Milsmann et al.[39], uses a phospholipid: detergent ratio of 0.625 and rapidly removes the detergent in a flow-through dialysis cell. The procedure forms a homogeneous population of single-layered vesicles with mean diameters of 50–100 nm.

ii. Column Chromatography

The formation of 100 nm single-layered phospholipid vesicles during removal of deoxycholate by column chromatography has been reported by Enoch and Strittmatter.[40] The method involves the treatment of phospholipid, in the form of either small sonicated vesicles or a dry lipid film, at a molar ratio of

deoxycholate to phospholipid of 1:2. Subsequent removal of the detergent during passage of the dispersion over a Sephadex G-25 column results in the formation of uniform 100 nm vesicles that are readily separated from small sonicated vesicles.

iii. Bio-Beads™

Another promising method for forming reconstituted membranes reported by Gerritsen et al.[Error! Reference source not found.] may also be applicable to LUV preparation. The system involves the removal of a nonionic detergent, Triton X-100, from detergent/phospholipid mixtures. This method is based on the ability of Bio-beads SM-2 to adsorb Triton X-100 selectively and rapidly. The dried lipid is suspended in 0.5–1.0% Triton X-100, and washed Bio-beads are added directly to the solution (about 0.3 g wet Bio-beads per mL of dispersion) and rocked for about 2 h at 4°C. The beads are removed by filtration The final particle size is determined by the conditions used including lipid composition, buffer composition, temperature and most critically, the amount and activity of the beads themselves.

d. Miscellaneous Methods

- **Slow Swelling in Non-electrolyte Solutions**

In 1969, Reeves and Dowben[41] reported a method for producing very large (up to several 10s of microns) single-layered liposomes by allowing a thinly spread layer of hydrated phospholipids to slowly swell in distilled water or a non-electrolyte solution. Typically, a mixture of lipids in ether or chloroform is deposited as a thin film on the bottom of a flat-bottomed beaker. The lipid is slowly hydrated by passing nitrogen gas saturated with water vapor over the film for several hours. When the film has completely hydrated, it will become opaque in appearance. Following hydration, distilled water or a non-electrolyte solution (e.g., sucrose) is carefully layered over the film and the beaker is placed in a 37°C water bath for several more hours. During this period very large single-walled vesicles are formed by a mechanism that begins with single bilayers swelling and budding from the film, pinching off and eluting into the aqueous medium. The yield of single-layered vesicles is high if conditions are right, but the main disadvantage of the technique is its sensitivity to any kind of mechanical agitation during vesicle formation. Also, since a very thin film is required and swelling times are long, this method would be difficult to scale up.

- **Removal of Chaotropic Ions**

Oku and MacDonald[43] developed a method of forming giant single-lamellar vesicles with diameters in the range of 10–20 microns by removal of sodium trichloroacetate by dialysis or dilution from a solution containing egg phospholipids and molar concentrations of sodium trichloroacetate. The yield of giant vesicles was critically dependent on the starting concentration of the chaotropic ion and temperature. Inclusion of a freeze-thaw step reduced the required concentration of trichloroacetate to about 0.1 M. The giant liposomes apparently were formed from concentrations of the ion that induced the transformation of phospholipids from the lamellar phase to the micellar phase. Other chaotropic ions were also shown to be effective, including urea or guanidine-HCl.

Methods for Controlling the Particle Size and Size Distribution of Liposomes

In most studies, using liposomes as drug carriers, particle size has not been rigorously controlled. In studies on tissue distribution reported to date, for example, various investigations have used either the initial liposome preparation containing a wide distribution of sizes (ranging from 0.2 to 10s of microns) or sonicated vesicles that, although exhibiting a narrow size distribution, are quite small and thus have a limited capacity to carry drugs. Judging from the few studies using controlled particle size, it is clear that vesicle size can have dramatic effects on the in vivo behavior of liposomes. Therefore, before liposome drug carrier systems can be taken seriously for pharmaceutical applications, their size will have to be controlled within reasonable limits. Three possible approaches have been explored for controlling the particle size distribution of liposome preparations:

1. Fractionation

Two methods have enjoyed widespread use for fractionating liposomes of the desired size from a heterogeneously sized population: centrifugation and size exclusion chromatography. Both can be used to enrich the product with the desired particle size but are limited in terms of the volumes that can be easily handled.

a. Centrifugation

Liposome sediment in a centrifugal field at a rate that is dependent on their size and density. Large liposomes composed of neutral lipids such as PC can easily be pelleted at fairly low g forces in a conventional centrifuge. Under proper conditions the smaller liposomes will remain in the supernatant. This method is useful for making gross cuts between small and larger liposomes but not for generating narrow particle size distributions. Also, the volumes that can be handled are limited by the volume capacity of the centrifuge. However, zonal rotors or continuous-flow centrifuges may be adaptable to this application. Another disadvantage to centrifugation is that liposomes smaller than about 0.5 micron tend to require high g forces and long spinning times in order to achieve effective separation from slightly larger particles in the 0.1–0.2 micron range. Also the capacity of the ultracentrifuges normally used for this purpose is limited to a few hundred mL per run.

b. Size Exclusion Chromatography

Column chromatography has been used for many years as an analytical method to assess the particle size of liposomes. Preparative scale chromatography has also been applied to produce liposomes of fairly homogeneous sizes. This method is particularly useful

for separating SUVs from larger structures. Typically, a column of Sepharose 4B is equilibrated with a buffer of the same osmolarity as the medium in which the vesicles were prepared and an aliquot of the liposomes is applied to the column. The column is eluted with the same buffer and fractions are collected. Large liposomes appear in the void volume, whereas SUVs elute with the included volume. Larger pore size chromatographic media, such as Sephacryl S1000, have been used in a similar fashion to fractionate populations of larger particles. In general, however, such chromatographic separations are quite limited in terms of volumes and throughput must be carried out in batches, resulting in significant dilution of the product.

2. Homogenization

In those cases in which a fairly small particle size is desirable, homogenization has proved a useful approach. In much the same way as milk is homogenized, the average particle size and polydispersity of vesicle dispersions can be reduced by passage through a high-pressure homogenizer. One such device marketed by Microfluidics Corp., Newton, MA, under the trade name Microfluidizer™ has been shown by Mayhew et al.[44] to generate vesicles in the 50–200 nm size range. Such homogenizers are amenable to scale up and throughput rates are high. As with other high-pressure devices, however, heat regulation can sometimes present problems and the shear forces developed within the reaction chamber can lead to partial degradation of the lipids. Another disadvantage relates to the empirical observation that conditions designed to produce approximately 200 nm particles often results in a bimodal distribution, with the bulk of the vesicles in the desired size range contaminated by a significant proportion of very small vesicles (less than 50 nm).

3. Capillary Pore Membrane Extrusion

A technique that has gained widespread acceptance for the production of liposomes of defined size and narrow size distribution, introduced by Olson et al.[45] in 1979, involves the extrusion of a heterogeneous population of fairly large liposomes through polycarbonate membranes under moderate pressures (100–250 psi). Such membranes have uniform straight-through capillary pores of defined size and polycarbonate does not bind liposomes containing charged species. This simple technique can reduce a heterogeneous population of MLVs or REVs to a more homogeneous suspension of vesicles exhibiting a mean particle size that approaches that of the pores through which they were extruded. MLVs with a mean diameter of 260 nm can be obtained following a single extrusion through 200 nm pore size polycarbonate membranes; 75% of the encapsulated volume resides in vesicles between 170 and 370 nm (as measured by negative stain electron microscopy). Upon additional extrusions through the same pore size membrane, the average size is reduced further, finally approaching about 190 nm with greater than 85% of the particles in the 170–210 nm range. Compared to SUV preparations this still represents a rather broad distribution of vesicle sizes, but compared to the original MLV population, which ranges in size from about 500 nm to several microns, it represents a considerable reduction of both average particle size and polydispersity. In practice, it is sometimes preferable to extrude sequentially through membranes of decreasing pore diameter. For example, a concentrated dispersion of MLVs may be difficult to extrude directly through a 200 nm pore size membrane under normal operating pressures (about 90 psi). It is advisable to begin the process by extrusion through a 0.8, 0.6, 0.4 and finally 0.2 micron pore size. Alternatively, it is possible use higher pressures to extrude concentrated dispersions through the smaller pore size membranes directly. A special high-pressure filter holder is required, however, since operating pressures may reach 250 psi. One such device is available commercially under the trade name LUVET™, which can accommodate up to 10 mL and is equipped with a recirculation mechanism, which permits multiple extrusion with little difficulty.

FREEZE-DRYING (LYOPHILIZATION)

Freeze-drying involves the removal of water from products in the frozen state at extremely low pressures. The process is generally used to dry products that are thermolabile and would be destroyed by heat-drying. Lyophilization has great potential as a method to solve long-term stability problems of liposomes. Intuitively, one would suspect that liposomes containing drugs entrapped in their bilayers would be better candidates for lyophilization than liposomes containing drugs entrapped in their aqueous compartments, since the lyophilization procedure would be expected to cause some bilayer disruption and subsequent leakage. Various studies have shown that water-soluble markers, such as carboxyfluorescein, do not survive freeze-drying in that even under the best of circumstances (use of saturated lipids and incorporation of cryoprotectants), a significant portion of the marker is lost on reconstitution. On the other hand, liposomes can retain > 90% of lipid-soluble drugs, such as Doxorubicin, on reconstitution. The amount retained depends on the use of cryoprotectants, lipid composition, liposome type, and loading dose. If the leaked-out drug is removed and the preparation frozen for a second time, essentially 100% of the drug is recoverable on reconstitution. This indicates that the original loss represents the portion of the drug residing in the aqueous compartment. Thus, when formulating, one must ensure that essentially all the drug is placed in the bilayer or accept a certain percentage of loss to the external medium. Recently, it was found that trehalose, a carbohydrate commonly found at high concentrations in organisms capable of surviving dehydration, is an excellent cryoprotectant for liposomes. It may work by stabilizing the bilayers, especially at their phase transition temperatures, during both freezing and thawing.[46]

LIPOSOME STABILITY

The stability of any pharmaceutical product is usually defined as the capacity of the formulation to remain within defined limits for a predetermined period of time (shelf-life of the product). The first step in designing any type of stability testing program is to specify these limits by establishing parameters defined in terms of chemical stability, physical stability and microbial stability. Next, methods must be established to evaluate each of these parameters. One must treat liposomal drug delivery systems in the same way as the more traditional pharmaceutical dosage forms are treated with respect to the establishment of clearly defined protocols for their characterization, manufacture, stability testing, and efficacy. Liposome stability is a complex issue and consists of physical, chemical and biological stability. In the pharmaceutical industry and in drug delivery, shelf life stability is also important. Physical stability indicates mostly the constancy of the size and the ratio of lipid to active agent. The cationic liposomes can be stable at 4°C for a long period of time, if properly sterilized.

A. Physical Stability

Stability of liposomes can be described by classical models from colloid science. Colloidal systems can be stabilized electrostatically, sterically, or electrosterically. In addition to normal colloids, self-assembling colloids can undergo other changes, such as fusion or phase change after aggregation. Liposome dispersion in a test tube exhibits a given physical and chemical stability. Generally, the former deals with the preservation of liposome structure while the latter one with the chemical structure of molecules. Therefore, physical stability means the preservation of liposome size distribution and the amount of the material encapsulated. Obviously this depends on mechanical properties of liposome membranes, their thermodynamics and colloidal properties of the system.

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 shelf 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 self-assembling 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 refers to molecular structure of liposomal components. (hydrolysis and oxidation of phospholipid) Physically stable formulations preserve both liposome size distribution and the amount of material encapsulated. The stability problem overcomes by using appropriate techniques like freezing, lyophilization and osmification. It is also prevented by using fresh solvents and freshly purified lipid, using inert nitrogen gas, avoid high temperature and include anti-oxidants like α-tocopherol.

B. Chemical Stability

Chemically, phospholipids are susceptible to hydrolysis. Additionally, phospholipids containing unsaturated fatty acids can undergo oxidative reactions. Much of the data on liposomes that have appeared in the literature can be considered suspect due to the use of phospholipids containing significant amounts of oxidation and hydrolysis products. These can cause dramatic changes in the permeability properties of liposomes. Preparative procedures (e.g., sonication, homogenization) or storage conditions (e.g., exposure to different pH values) can affect the decomposition rate of the liposomal lipids.

1. Lipid Peroxidation

Most of the phospholipid liposomal dispersions used contain unsaturated acyl chains as part of the molecular structure. These chains are vulnerable to oxidative degradation (lipid peroxidation). The oxidation reactions can occur during preparation, storage, or actual use. Oxidative deterioration of lipids is a complex process involving free-radical generation and results in the formation of cyclic peroxides and hydroperoxides. Oxidation of the phospholipids may be minimized by a number of methods:

1. Minimum use of unsaturated phospholipids (if appropriate)
2. Use of argon or nitrogen to minimize exposure to O_2
3. Use of light-resistant containers
4. Removal of heavy metals (EDTA)
5. Use of antioxidants such as a-Tocopherol or BHT

2. Lipid Hydrolysis

The most important degradation product resulting from lecithin hydrolysis is lyso-lecithin (lyso-PC), which results from hydrolysis of the ester bond at the C_2 position of the glycerol moiety. Many workers choose the formation of lyso-PC as a standard measure for the chemical stability of phospholipids, since the presence of lyso-PC in lipid bilayers greatly enhances the permeability of liposomes. It is, therefore, extremely important that the formation of lyso-PC be kept to a minimum during storage. Lyso-PC is usually analyzed by phospholipid extraction followed by separation of PC and lyso-PC by TLC. Although factors such as sonication could affect the degree of lyso-PC formation, probably the single most important method of minimizing this problem is by the proper sourcing of the phospholipids to be used. They should be essentially free of any lyso-PC to start with and, of course, be free of any lipases.

3. Miscellaneous Chemical Stability Concerns

One must not ignore the fact that the other bilayer lipids, which may be present, can also decompose. For example, cholesterol, in aqueous dispersion, has been shown to oxidize rapidly when unprotected. Finally, the drug itself must be considered. The stability profile of the "free"

drug may be quite different from its profile in the encapsulated state. In fact, a number of strategies have been developed that are based on protecting drugs from biological environments by encapsulating them in liposomes. Examples include the protection of insulin from proteolytic enzymes of the gastrointestinal tract and the prolongation of ester hydrolysis of prodrugs (e.g., cortisone hexadecanoate) after intramuscular administration.

C. Stability of Liposomes in Biological Fluids

The ultimate efficacy of a liposomal dosage form will be judged by the ability of the formulator to reliably control the amount of free drug that reaches the site of action over a given period of time. Generally, the exact "site of action" or receptor site at the molecular level is not known and one relies on attaining reproducible blood levels of the drug. With traditional nonparenteral dosage forms, only the free drug is absorbed and once the drug is in the bloodstream, it has no memory about where it came from. Thus, the only method available to control the pharmacokinetics of a drug is to adjust the amount of drug that enters the blood as a function of time. Parenteral, especially intravenous, administration of liposomally encapsulated drugs presents the formulator with additional means to control the pharmacokinetics of the drug. Factors that affect the pharmacokinetics of parenteral liposome administration include

1. Concentration of free drug in blood
2. Concentration of liposomes and their entrapped drug in blood
3. Leakage rate of drug from the liposome in the blood
4. Disposition of the interact drug-carrying liposomes in the blood

In order to reliably control the pharmacokinetics of these complex systems, one must be able to separate the

1. Stability (leakage rate) of drug from the liposome in the blood.
2. Disposition of the intact drug-carrying liposome in the blood. The pharmacokinetics of intact liposomes is beyond the scope of this chapter and has been thoroughly reviewed elsewhere.[47]

A). Liposome Stability in Blood and Plasma

The inability of liposomes to retain entrapped substances, when incubated with blood or plasma, has been known for about a decade. The fact that high molecular weight substances, such as inulin and even albumin, leak out on incubation with plasma suggests that more than superficial damage is being done to the liposomes even though their gross morphology appears unchanged. The instability of liposomes in plasma appears to be the result of the transfer of bilayer lipids to albumin and high-density lipoproteins (HDLs). Additionally, some of the protein is transferred from the lipoprotein to the liposome. Both lecithin and cholesterol also exchange with the membranes of red blood cells. Liposomes are most susceptible to HDL attack at their gel to liquid crystalline phase transition temperature. It is, therefore, worthwhile to determine by differential scanning calorimetry whether the formulation has a phase transition temperature close to 37°C. The susceptibility of liposomal phospholipid to lipoprotein and phospholipase attack is strongly dependent on liposome size and type. Generally, MLVs are most stable, since only a portion of the phospholipid is exposed to attack, and SUVs are the least stable because of the stresses imposed by their curvature. Liposomes prepared with higher chain length phospholipids are most stable both in buffer and in plasma. Incorporation of charged lipid into the bilayer decreases stability in plasma even when cholesterol is included to bring the liposomes to the gel state. Cholesterol and sphingomyelin are generally very effective in reducing the instability of liposomes in contact with plasma. It is believed that the primary reason for this effect is not the increased bilayer tightness produced by cholesterol but the prevention of transfer of phospholipid to the plasma lipoprotein and red blood cell membrane.

B). Stealth® Liposomes

One of the main disappointments of drug delivery using conventional liposomes was the realization that neither mechanical nor electrostatic stabilization can increase liposome stability in biological systems, such as in blood circulation. This is a very "liposomicidal" environment, because the body protects itself with an elaborate immune system and liposomes, if not quickly degraded by lipoproteins, are rapidly recognized as foreign particles and quickly taken up by the phagocytic cells of the body's immune system, which are located mostly in the liver and spleen. A major breakthrough in liposome application was the realization that external steric stabilization can increase liposome stability in a biological environment. It was discovered that covering liposomes with hydrophilic, nonionic polymers greatly increased their stability in blood circulation.[3,48-49] They must be inert, well solvated and compatible with the solvent and have polarizability close to that of water. Steric stabilization can be induced by the surface attachment of various natural or synthetic polymers, either by adsorption, hydrophobic insertion, electrostatic binding, or, preferably, by grafting via covalent bond. Nonionic, water-compatible, flexible, and well-hydrated polymers are preferred. The repulsion between surfaces with attached polymers was shown to be dependent on the grafting density and degree of polymerization. Normally, liposome bilayers contain 5 mol% of lipid with covalently attached polyoxyethylene glycol with molecular weight of 2,000 Da. Neither introduction of a larger amount of PEG-lipid, nor longer polymer chains resulted in an improved stability in circulation. This is due to increased lateral pressure of the polymer above the surface and increased aqueous solubility of these large molecules. Normally, distearoyl chains are used to increase the anchoring effect. Qualitatively one can explain the enhanced stability of such sterically stabilized liposomes in liposomicidal environments by their ability to prevent adsorption of various blood

components and their close approach.[2] It was proposed that the major mechanism of liposome uptake and disintegration in plasma is reaction with proteins of the immune system, which adsorbs onto foreign colloidal particles and tags them for subsequent macrophage uptake.[2,49] It is readily assumed that in the presence of surface-attached polymer, the adsorption of immunoglobulins or proteins of the complement cascade onto liposomes are reduced and that lipid exchange interactions, which deplete liposome lipids, are minimized.

C). Liposome Stability in the Gastrointestinal Tract

Although many papers have been published on the oral administration of liposomally encapsulated drugs, especially insulin, very little effort has been made to critically assess the stability of liposomes in the environment of the gastrointestinal tract. Even distearoylphosphatidylcholine/cholesterol liposomes are very unstable in the gastrointestinal tract and liposomally encapsulated and free drug give about the same pharmacokinetics when administered by the oral route to rats.

STABILITY TESTING (GENERAL CONSIDERATIONS)

Stability testing of liquid disperse systems is one of the most difficult problems faced by formulation chemists. The scientist is often asked to predict the shelf-life of a product or choose between experimental formulations based on estimates of how well they will hold up with time. There are no standardized tests available to determine physical stability and quite often there is no certainty of what type of stability is being investigated. The first order of priority for solving stability problems of disperse systems is to define clearly the type or types of stability of concern. The testing is most likely to yield information applicable to the estimation of the product's shelf-life. Stability tests commonly stress the system to limits beyond those that the product will ever encounter. Typical examples of stress tests include exposure of the product to high temperatures. It is important to understand whether these tests are being performed because the product is expected to encounter these conditions or because, even though these conditions will never be approached, the results will help predict shelf-life at more moderate conditions. High-temperature testing (> 25°C) is almost universally used for heterogeneous products. Various laboratories store their products at temperatures ranging from 4°C (refrigerator temperature) to 50°C (or perhaps even higher). The temperatures used in heat-cool cycling are also quite varied, often without regard for the nature of the product.

For liposomes, elevated temperatures may dramatically alter the nature of the interfacial film, especially if the phase transition temperature is reached. If one expects the product to be exposed to a temperature of 45°C for extended period of time or for short durations, (shipping and warehouse storage), studies at 45–50°C, (long-term and heat-cool cycling), are quite justified. A study of a product at these temperatures determines.

(1) How the product is holding up at this elevated temperature; and

(2) Whether the damage is reversible or irreversible when the product is brought back to room temperature.

If temperatures higher than the system will ever encounter are used, even in short-term heat-cool cycling, there is a risk of irreversibly damaging the bilayers so that when it is brought back to room temperature, the membrane cannot heal. If a liposomal dispersion is partially frozen and then thawed, ice crystals nucleate and grow at the expense of water. The liposomes may then be pressed together against the ice crystals under great pressure. If the crystal grows to a size greater than the void spaces, instability is more likely. That is why a slower rate of cooling, resulting in larger ice crystals, produces greater instability. Polymers may retard ice crystal growth Van Bommel and Crommelin[50] showed that even one freeze-thaw cycle causes almost complete rapid leakage of carboxyfluorescein from liposomes (REVs) prepared from unsaturated phospholipids (even when cholesterol is added). However, liposomes composed of distearoylphosphatidylcholine, dipalmitoylphosphatidylglycerol and cholesterol show slightly better freeze-thaw stability. Stability testing protocols should be developed for liposomal products on a case-by-case basis. A typical protocol for a product that would be shipped in vehicles not equipped with climate control and stored in warehouses for prolonged periods under similar conditions might include testing under the following conditions:

1. One month at 45°C
2. One month at 4°C
3. Six months at 37°C
4. 12–24 months at room temperature
5. 12–24 months at various light intensities
6. Two to three “freeze-thaw” cycles (-20°C 25°C)
7. Six to eight “heat-cool” cycles (5°C 45°C, 48 hours at each temperature)
8. 24–48 h on a reciprocating shaker at 60 cycles/min (estimates transportation conditions).

One should be certain that studies are performed using all types and sizes of containers. Under each of the test conditions, the following data can be collected:

1. Visual and microscopic observations, e.g., flocculation
2. Particle size profiles
3. Rheological profiles
4. Chemical stability
5. Extent of leakage

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 disorders

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.

MEDICAL APPLICATIONS.

Due to their biocompatibility, biodegradability and colloidal properties, liposomes are one of the most studied drug delivery systems. They can act as a disperser for drugs that are difficult to solubilize, a sustained release system for microencapsulated agents, penetration enhancers and site-specific delivery vehicles. In addition, in many cases the toxicity of the encapsulated drug is reduced because liposomes do not accumulate in some organs, such as the heart and kidneys.[51] While drug-laden liposomes can be applied via all administration routes, including parenteral, topical, oral, and pulmonary, systemic applications are the most widely used. The major problem is the rapid leakage of the encapsulated drugs and quick uptake of liposomes by the cells of the reticuloendothlial system (RES).[7,49] This natural fate of liposomes can be used to target these cells resulting in major improvements of the therapeutic index of drugs in the treatment of parasitic infections of these cells.[52-55] Recently, sterically stabilized liposomes, which can evade rapid clearance by the cells of RES, were developed and improvements in anticancer therapy in animal models[7,56-59] as well as in human patients were reports[7,60-61] The therapeutic promise of liposomes as a drug delivery system is fast becoming a reality. One must keep in mind that only in the last 15 years or so have real advances been made in translating the progress from university laboratories into pharmaceutically acceptable dosage forms. Pharmaceutical scientists collaborating with process engineers have been able to produce large volumes of sterile, pyrogen-free liposomes with acceptable shelf-lives. With current emphasis on increasing therapeutic indices of drugs it appears quite likely that these biocompatible, biodegradable vehicles will continue to receive increased attention from the pharmaceutical industry. As of this printing, more than 10 companies plan to or have applied to the Food and Drug Administration for approval to test approximately 20 liposomally entrapped drug products. These dosage forms include anticancer and antifungal agents as well as drugs to combat arthritis, glaucoma and dry eye. Within a short period of time one might expect to see a broad range of liposomal products in various stages of clinical testing. The most promising appear to be liposomal products specifically formulated to facilitate the following:

1. Site-Specific Delivery

Particular emphasis has been placed on disease states involving the RES. Examples include antimonial compounds for parasitic disease, immunomodulation using macrophage activating agents and antiviral treatment using ribavirin.

2. Site-Avoidance Delivery

The most promising examples are liposomal Doxorubicin (reduced cardiotoxicity) and liposomal Amphotericin B (reduced nephrotoxicity).

3. Sustained or Controlled Release

Examples include inhalation of bronchodilators, ocular delivery of antibiotics, intramuscular delivery of peptides and topical delivery of a variety of drugs.

4. Passive targeting

Small particles can extravasate at the sites where the vascular system is leaky. This often happens in tumors and sites of trauma, either due to badly formed blood vessels in fast-growing tumors or due to the healing process itself. The accumulation in these sites is proportional to blood circulation times and long-circulating liposomes were shown to accumulate in tumors.[7, 62] Still on a laboratory scale, are attempts of active targeting of (drug-laden) liposomes with attached ligands. Chemistry of attachment of various ligands to liposome surface or to the terminus of surface-grafted polymers was developed by Hansen et al.[52]

5. Gene therapy

Conventional liposomes have also been tried as delivery systems to deliver DNA into cells.[62-644] The rationale was the ability of liposomes to enhance intracellular accumulation, i.e., facilitate transfer of these large and heavily charged molecules across rather impermeable cell membranes. The procedures were rather cumbersome and inefficient, especially in the encapsulation of larger fragments. Because the technique was applicable in practice only *in vitro* or *ex vivo* and required addition of fusogenic agents, its use rapidly declined with the emergence of electroporation. Improvements achieved by using pH-sensitive liposomes

did not change the trend. Another candidate for DNA delivery is the virosome, a liposome containing fusogenic viral proteins. The problem with such structures is effective DNA encapsulation. Even with a possible addition of condensing agents DNA particles are still rather large and not easy to encapsulate into relatively small liposomes and virosomes. Because these structures contain viral or bacterial proteins, immunogenicity may be a problem as well. The use of liposomes in transfection to deliver a gene that encodes a particular protein into appropriate cells, with the aim of inducing the production of the encoded protein by the targeted cell, was revived with the emergence of cationic liposome-nucleic acid complexes. Liposomes seem to be favored with respect to viral vectors because of the larger carrying capacity, apparent lack of immunogenicity and for safety reasons. Efficient transfection can be achieved either by using supercoiled or linear plasmids. Their size varies but the range is normally between half and several tens of kilobase pairs. Following difficulties to transfect cells with "DNA-micelle" complexes, bilayer forming diacyl cationic lipids are primarily used for transfection.

NONMEDICAL APPLICATIONS OF LIPOSOMES

Liposomes make very useful models for studying biomembranes and membrane proteins. In addition to applications in basic sciences and medical applications, their properties can be exploited for various other uses. Because hydrophobic substances can be dispersed in a hydrophilic medium they can be used in the food industry. The best known example is microencapsulated enzymes in cheese ripening. Liposomes can be used as a signal carrier in diagnostics. Normally ligands contain one marker group (fluorescent or radioactive). If ligands are conjugated to liposomes, which can contain hundreds of markers, the signal can be accordingly amplified. Other uses involve the use of liposomes in the coating industry and ecology. It was shown that bioreclamation can be improved if nutrients and bacteria are dispersed in/with liposomes. Liposomes, however, have achieved the largest impact in cosmetics. Hundreds of products exist, ranging from skin creams, to after-shaves, to body lotions, to sunscreens. Formulations exist from simple liposome kits with which customers can mix in their own ingredients to sophisticated formulations with encapsulated enzymes and antibiotics. While real benefits are debated, we can simply state that liposomes offer a biocompatible, natural and water-based system to solubilize hydrophobic ingredients in what seems more appropriate than the use of detergents, oils, or alcohols in nonliposomal formulations. There is little doubt that with improved understanding of liposome stability and interaction characteristics, many other successful applications will follow.

Industrial Production of Liposomes[7-8]

The several preparation methods described in the literature, only a few have potential for large scale manufacture of liposomes. The main issues faced to formulator and production supervisor are presence of organic solvent residues, physical and chemical stability, pyrogen control, sterility, size and size distribution and batch to batch reproducibility. Liposomes for parenteral use should be sterile and pyrogen free. For animal experiments, adequate sterility can be achieved by the passage of liposomes through up to approximately 400 nm pore size Millipore filters. For human use, precautions for sterility must be taken during the entire preparation process: that is,

1) The raw materials must be sterile and pyrogen free,
2) Preparation in sterile system: working area equipped with laminar flow and
3) Use of sterile containers

Some issues related to phospholipids need attention. The liposomes based on crude egg yolk phospholipids are not very stable. The cost of purified lipids is very high. Recently, liposomes have been prepared using synthetic and polymerizable lipids. The liposomes prepared from polymerizable phospholipids are exposed to UV light. The polymerization process takes place in the bilayer(s). Such liposome preparations usually have better storage stability. It should be noted that such materials usually are phospholipid analogues and their metabolic fates have yet to be established.

Application in Medicine

As of 2016, 11 drugs with liposomal delivery systems have been approved and 11 additional liposomal drugs were in clinical trials.

Table 2: List of clinically-approved liposomal drugs

Name	Trade name	Lipid composition	Company	Indication
Liposomal amphotericin B	Abelcet	DMPC: DMPG	Enzon	Fungal infections
Liposomal amphotericin B	Ambisome	HSPC: DSPG: Cholesterol	Gilead Sciences	Fungal and protozoal infections
Liposomal cytarabine	Depocyt	Cholesterol: triolein, :DOPC: DPPG	Pacira (formerly SkyePharma)	Malignant lymphomatous Meningitis
Liposomal daunorubicin	DaunoXome	DSPC: Chol	Gilead Sciences	HIV-related Kaposi's sarcoma
Liposomal doxorubicin	Myocet	EPC and cholesterol	Zeneus	Combination therapy with cyclophosphamide in metastatic breast cancer

Liposomal morphine	DepoDur	DOPC:cholesterol: DPPG: tricaprylin: triolein	Skye Pharma, Endo	Postsurgical analgesia
Liposomal verteporfin	Visudyne	EPG, DMPC	QLT, Novartis	Age-related macular degeneration, pathologic myopia, ocular histoplasmosis
Liposome-PEG Doxorubicin	Doxil/Caelyx	PEGDSPE:HSPC: Cholesterol	Ortho Biotech, Schering-Plough	HIV-related Kaposi's sarcoma, metastatic breast cancer, metastatic ovarian cancer
DaunoXome	Daunorubicin	DSPC,Cholesterol	NeXstar, USA	Kaposi's sarcoma, breast & lung cancer
Amphotec	Amphotericin-B	Cholesteryl sulfate	SEQUUS, USA	fungal infections, Leishmaniasis
Nyotran	Nystatin	DMPC, DMPG	Aronex Pharm	Systemic fungal infections
Onivyde	Irinotecan	DSPC, Cholesterol, mPEG-DSPE 2000	Merrimack Pharmaceuticals, Inc.	metastatic adenocarcinoma of the pancreas

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