LIPOSOMES: AS TARGETED DRUG DELIVERY SYSTEM

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India.

The need to deliver drugs specifically to targets within the body was recognized early last century (1906) by Paul Ehrlich. Drugs administered as such are known to exhibit a variety of toxic effects because of their nonspecific distribution in the body, acting on diseased and healthy tissues alike. Targeting of drugs was initially attempted by the use of antibodies (O’Neill 1979). It was thought that linking of a cytotoxic drug to an antibody raised against a cell type would deliver the drug to that cell. However, success in tumour bearing animals and humans was only modest and it had to await the development of monoclonal antibodies to obtain promising clinical results.

Prior to this, work with polyclonal antibodies coincided with increased interest in drug delivery research in the 1970s and 1980s by the use of lactic/glycolic polymers, dextran, nylon capsules, lectins, glycoproteins, desoxyribonucleic acid and cells such as erythrocytes, neutrophils and fibroblasts. Many such systems, however, fell by the wayside because of a variety of reasons including short or long-term toxicity, lack of biodegradability, unfavourable in vivo bio-distribution and poor system availability. Arguably, systematic work on drug delivery only began in 1970 at the Royal Free Hospital School of Medicine on the author’s joining the laboratory of the late Brenda Ryman to work on liposomes. Liposomes serves as a novel drug delivery system, the „Nano medicines“ according to today’s parlance. Work began in September 1970 and because of my background in animal work at the cellular, subcellular and molecular level, we were able to establish within months a number of facts that were crucial in us pursuing liposomes seriously as a universal delivery system. For instance, liposomes were found to entrap small and large pharmacologically active molecules that were quantitatively retained by the carrier in the circulating blood. Further, liposomes injected into animals by the intravenous, intramuscular or intraperitoneal route were tolerated and appeared to be nontoxic. Importantly, this early work also
demonstrated that liposomal fate in vivo could be controlled in terms of rate of clearance from the blood circulation and uptake by tissues.

INTRODUCTION

Liposomes are small artificial vesicles of spherical shape that can be created from cholesterol and natural non-toxic phospholipids. Due to their size and hydrophobic and hydrophilic character (besides biocompatibility), liposomes are promising systems for drug delivery. Liposome properties differ considerably with lipid composition, surface charge, size, and the method of preparation (Table 1). Furthermore, the choice of bilayer components determines the ‘rigidity’ or ‘fluidity’ and the charge of the bilayer. For instance, unsaturated phosphatidylcholine species from natural sources (egg or soybean phosphatidylcholine) give much more permeable and less stable bilayers, whereas the saturated phospholipids with long acyl chains (for example, dipalmitoylphosphatidylcholine) form a rigid, rather impermeable bilayer.

It has been displayed that phospholipids impulsively form closed structures when they are hydrated in aqueous solutions. Such vesicles which have one or more phospholipid bilayer membranes can transport aqueous or lipid drugs, depending on the nature of those drugs. Because lipids are amphipathic (both hydrophobic and hydrophilic) in aqueous media, their thermodynamic phase properties and self-assembling characteristics influence en tropically focused confiscation of their hydrophobic sections into spherical bilayers. Those layers are referred to as lamellae. Generally, liposomes are definite as spherical vesicles with particle sizes ranging from 30 nm to several micro meters. They consist of one or more lipid bilayers surrounding aqueous units, where the polar head groups are oriented in the pathway of the interior and exterior aqueous phases. On the other hand, self-aggregation of polar lipids is not limited to conventional bilayer structures which rely on molecular shape, temperature, and environmental and preparation conditions but may self-assemble into various types of colloidal particles.

Liposomes are extensively used as carriers for numerous molecules in cosmetic and pharmaceutical industries. Additionally, food and farming industries have extensively studied the use of liposome encapsulation to grow delivery systems that can entrap unstable compounds (for example, antimicrobials, antioxidants, flavours and bioactive elements) and shield their functionality. Liposomes can trap both hydrophobic and hydrophilic compounds,
avoid decomposition of the entrapped combinations, and release the entrapped at designated targets.

Because of their biocompatibility, biodegradability, low toxicity, and aptitude to trap both hydrophilic and lipophilic drugs and simplify site-specific drug delivery to tumour tissues, liposomes have increased rate both as an investigational system and commercially as a drug-delivery system. Many studies have been conducted on liposomes with the goal of decreasing drug toxicity and/or targeting specific cells.

Liposomal encapsulation technology (LET) is the newest delivery technique used by medical investigators to transmit drugs that act as curative promoters to the assured body organs. This form of delivery system proposal targeted the delivery of vital combinations to the body. LET is a method of generating sub-microscopic foams called liposomes, which encapsulate numerous materials. These ‘liposomes’ form a barrier around their contents, which is resistant to enzymes in the mouth and stomach, alkaline solutions, digestive juices, bile salts, and intestinal flora that are generated in the human body, as well as free radicals. The contents of the liposomes are, therefore, protected from oxidation and degradation. This protective phospholipid shield or barrier remains undamaged until the contents of the liposome are delivered to the exact target gland, organ, or system where the contents will be utilized.

Clinical medication keeps an enormously broad range of drug molecules at this time in use, and new drugs are added to the list every year. One of the main aims of any cure employing drug is to increase the therapeutic index of the drug while minimizing its side effects. The clinical usefulness of most conservative chemotherapeutics is restricted either by the incapability to deliver therapeutic drug concentrations to the target soft tissue or by Spartan and harmful toxic side effects on normal organs and tissues. Different approaches have been made to overcome these difficulties by providing the ‘selective’ delivery to the target area; the ideal solution would be to target the drug alone to those cells, tissues, organs that are affected by the disease. Selected carriers, for instance colloidal particulates and molecular conjugates, can be appropriate for this determination. Colloidal particulates result from the physical incorporation of the drug into a particulate colloidal system, for instance reverse micelles, noisome, micro- and nano-spheres, erythrocytes, and polymers and liposomes. Among these carriers, liposomes have been most studied. Their attractiveness lies in their composition, which makes them biodegradable and biocompatible. Liposome involves an
aqueous core entrapped by one or more bilayers composed of natural or synthetic lipids. They are composed of natural phospholipids that are biologically inert and feebly immunogenic, and they have low inherent toxicity. Furthermore, drugs with different lipophilicities can be encapsulated into liposomes: strongly lipophilic drugs are entrapped almost totally in the lipid bilayer, intensely hydrophilic drugs are located entirely in the aqueous compartment, and drugs with intermediary logP effortlessly partition between the lipid and aqueous phases, both in the bilayer and in the aqueous core.

The present review will briefly explain the characteristics of liposomes and explore the related problems and solutions proposed, with a focus on liposome preparation, characterizations, affecting factors, advantages, and disadvantages. In particular, we return to the literature relating to high-stability, long-circulating liposomes (stealth liposomes), and their field of application.

The general problem of targeted drug transport is critically reviewed and three principle components of targeted systems are discussed: the target, the vector molecule, and the carrier. Different systems of drug targeting are briefly described: local drug application, chemical modification of the drug molecule, physical targeting under the action of pH, temperature, or magnetic field. The idea of a vector molecule is discussed and different methods of vector molecule coupling with the drug are reviewed (direct coupling, coupling via spacer group or polymer molecule, etc.).

It is shown that the most promising approach seems to be the use of a drug-containing micro container with the vector molecule immobilized on its outer surface. Different types of micro containers are briefly described: microcapsules, cell hosts, and liposomes. The advantages of liposomes as drug containers are shown and the main problems of their use for drug targeting in vitro and in vivo conditions are discussed. One of the most important problems is the problem of vector molecule immobilization on liposome surfaces. The principle four different immobilization methods: adsorption, incorporation, covalent binding, and hydrophobic binding. Targeted liposome transport is described in model systems, cell cultures, and experimental animals. It is shown that targeted liposomes may release a drug via diffusion, lysis, or endocytosis by appropriate cells. The problems of targeted liposome technology and clinical application are analyzed.
Basic Structure
The outer part of a liposome, the membrane, is composed of a phospholipid bilayer enclosing in an aqueous volume. Phospholipids are the main building block of liposomes. They have tubular shape and two acyl chains attached to a polar head, which with hydration results in a bilayer, with a hydrophilic head and two hydrophobic tails. This combination means that liposomes are amphiphilic. Liposomes can either be naturally derived phospholipids or of pure surfactant components like DOPE.

Cholesterol is important for liposomes as it is used as a membrane additive to fill up the empty spaces between the phospholipids. Cholesterol increases the fluidity of the cell’s membrane and provides an increase in the order of the bilayer as it anchors the components of the bilayer more strongly. This increases the transition temperature of the system and provides stability.

<table>
<thead>
<tr>
<th>Advantages Of Liposome</th>
<th>Disadvantages Of Liposome</th>
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<tbody>
<tr>
<td>Liposomes increased efficacy and therapeutic index of drug (actinomycin-D)</td>
<td>Low solubility</td>
</tr>
<tr>
<td>Liposome increased stability via encapsulation</td>
<td>Short half-life</td>
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<tr>
<td>Liposomes are non-toxic, flexible, biocompatible, completely biodegradable, and non-immunogenic for systemic and non-systemic administrations</td>
<td>Sometimes phospholipid undergoes oxidation and hydrolysis-like reaction</td>
</tr>
<tr>
<td>Liposomes reduce the toxicity of the encapsulated agent (amphotericin B, Taxol)</td>
<td>Leakage and fusion of encapsulated drug/molecules</td>
</tr>
<tr>
<td>Liposomes help reduce the exposure of sensitive tissues to toxic drugs</td>
<td>Production cost is high</td>
</tr>
<tr>
<td>Site avoidance effect</td>
<td>Fewer stables</td>
</tr>
<tr>
<td>Flexibility to couple with site-specific ligands to achieve active targeting</td>
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Classification of Liposomes

The liposome size can vary from very small (0.025 μm) to large (2.5 μm) vesicles. Moreover, liposomes may have one or bilayer membranes. The vesicle size is an acute parameter in determining the circulation half-life of liposomes, and both size and number of bilayers affect the amount of drug encapsulation in the liposomes.

On The Basis Of Their Size and Number of Bilayers

Table 1: Liposome Classification Based On Structural Features.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MLV</td>
<td>Multilamellar large vesicles</td>
</tr>
<tr>
<td>OLV</td>
<td>Oligolamellar vesicles</td>
</tr>
<tr>
<td>UV</td>
<td>Unilamellar vesicles</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
</tr>
<tr>
<td>MUV</td>
<td>Medium sized unilamellar vesicles</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicles</td>
</tr>
<tr>
<td>GUV</td>
<td>Giant unilamellar vesicles</td>
</tr>
<tr>
<td>MVV</td>
<td>Multivesicular vesicles</td>
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Table 2: Liposome Classification Based on Method of Liposome Preparation.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>REV</td>
<td>Single or oligolamellar vesicle made by reverse phase evaporation method.</td>
</tr>
<tr>
<td>MLV / REV</td>
<td>Multilamellar vesicles made by reverse phase evaporation method.</td>
</tr>
<tr>
<td>SPLV</td>
<td>Stable plurilamellar vesicles</td>
</tr>
<tr>
<td>FATMLV</td>
<td>Frozen and thawed</td>
</tr>
<tr>
<td>MLV VET</td>
<td>Vesicles prepared by extrusion method.</td>
</tr>
<tr>
<td>FUV</td>
<td>Vesicles prepared by fusion</td>
</tr>
<tr>
<td>FPV</td>
<td>Vesicles prepared by French press</td>
</tr>
<tr>
<td>DRV</td>
<td>Dehydration- rehydration vesicles</td>
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</tbody>
</table>

Mechanism of Liposome Formation

Liposomes are vesicular structures consisting of hydrated bilayers. Liposomes structures used for pharmaceutical purposes consist of a phospholipid backbone. But other classes of molecules can form bilayer based vesicular structures as well. On the other hand not all the hydrated phospholipids form bilayer structures. Other forms of self aggregation such as inverted hexagonal phases or micelles with completely different properties can occur. The common feature that all bilayer forming compounds share is their amphilicity. They have defined polar and nonpolar regions. In water the hydrophobic regions tend to self aggregate and the polar regions tend to be in contact with the water phase. Israelachvili and coworkers defined critical packing parameter p by

\[ P = \frac{v}{a_0 l_c} \]
Where \( v \) is the molecular volume of the hydrophobic part, \( a_0 \) is the optimum surface area per molecule at the hydrocarbon water interface, and \( l_c \) is the critical half thickness for the hydrocarbon region which must be less than the maximum length of the extended lipid chains.

For \( p < 1/3 \), spherical micelles are formed. In this category fall single chain lipids with large head group areas, e.g., lysophosphatidylcholine.

For \( 1/3 < \frac{1}{2} \) globular or cylindrical micelles are formed. Double chain “fluid state” lipids with large head area \((1/2 < p < 1)\) form bilayers and vesicles.

This occurs also with double chain “gel state” lipids with small head groups and \( p \approx 1 \). For \( p > 1 \) inverted structures such as the inverted hexagonal phase can be observed. An additional condition required for bilayer formation is that the compound can be classified as a nonsoluble swelling amphiphile.

**Raw Materials for Formation of Liposomes**

Liposomes that are used as carriers for drugs or diagnostic agents should be prepared from constituents that are safe for use in humans. Although limited experience is available on the safety of liposomes, phosphatidylcholines and phosphatidylglycerols from natural sources, semisynthetically or fully synthetically produced and cholesterol and PEGylated phosphatidylethanolamine, are frequently encountered in liposomes designed as drug carriers for parenteral administration or for in vivo diagnostic purposes. Phosphatidylcholine (PC) is routinely used as a bulk neutral phospholipid. As a negatively charged lipid, phosphatidylglycerol (PC) is often selected. Finally, if it is desirable to reduce the permeability of “fluid crystalline state” bilayers, cholesterol is added to bilayer structure. Sometimes lipids with a special affinity for certain target cells in the body are deliberately inserted in bilayer. This was, for instance, the case when hepatocytic delivery was aimed for and lactosylceramide, a ligand with a special affinity for hepatocytes, was included in the liposomal bilayer.

Five groups of phospholipids that can be used for the liposomal preparation can be discerned

1. Phospholipids from natural sources
2. Modified natural phospholipids
3. Semisynthetic phospholipids
4. Fully synthetic phospholipids
5. Phospholipids with non-natural head groups

**Phospholipids From Natural Sources**

The sources for natural phospholipids, mainly PC, but also phosphatidylethanolamine (PE), phosphatidylinositol (PI), and sphingomyelin (SPM), are egg yolks and soybeans. These PC’s are mixed acyl ester phospholipids. Apart from source dependent differences in acyl chain type, considerable interbatch variation has been observed for egg PC. The esterified acyl chains of egg PC are different from those of soybean PC.

**Modified Natural Phospholipids**

Natural phospholipids can be modified. Because of their degree of unsaturation, which makes them sensitive to oxidation, PC from natural sources can be catalytically hydrogenated. Partially or fully hydrogenated natural PCs are readily available. The iodine value of these lipids is reduced as the number of unsaturated C=C bonds drops. Dependent on the degree of unsaturation left after the hydrogenation process, phase transition temperatures can be identified for liposomal dispersions of the partially hydrogenated PCs. Head group modifications can be performed by using phospholipase. With this enzyme one can convert PC into PG, PE or phosphatidylyserine (PS).

**Phospholipids With Nonnatural (Head) Groups**

The idea of maintaining the fate of liposomes in the body by selecting the appropriate bilayer characteristics has led to modified phospholipids. The circulation time of liposomes in the blood compartment can be considerably prolonged when polyethyleneglycol chains are attached to bilayer constituents.

Alternatively, for physically widely different structures, such as monoclonal antibodies or just a simple peptide. PEG has been linked to PE for the preparation of long circulating liposomes. Various reaction schemes have been developed. Molecular weights fractions for maximum prolongation of circulation times for PEG vary between 1900 and 5000. Allen and co-workers described the synthesis of a PEG-carbonate derivative of PE. Klibanov et al. used a succinidyl conjugation method, while Blume and Cevc adopted the procedure that Abuchowski and co-workers described for the preparation of PEG-albumin conjugates.
METHODS OF LIPOSOME PREPARATION

General methods of preparation
All the methods of preparing the liposomes involve four basic stages:
1. Drying down lipids from organic solvent.
2. Dispersing the lipid in aqueous media.
3. Purifying the resultant liposome.
4. Analyzing the final product.

Method of Liposome Preparation And Drug Loading
The following methods are used for the preparation of liposome:
1. Passive loading techniques
2. Active loading technique.

Passive loading techniques include three different methods:
1. Mechanical dispersion method.
2. Solvent dispersion method.

Mechanical Dispersion Method
The following are types of mechanical dispersion methods:
1.1. Sonication.
1.2. French pressure cell: extrusion.
1.3. Freeze-thawed liposomes.
1.4. Lipid film hydration by hand shaking, non-hand. Shaking or freeze drying.
1.5. Micro-emulsification.
1.6. Membrane extrusion.
1.7. Dried reconstituted vesicles.

Sonication
Sonication is perhaps the most extensively used method for the preparation of SUV. Here, MLVs are sonicated either with a bath type sonicator or a probe sonicator under a passive atmosphere. The main disadvantages of this method are very low internal volume/encapsulation efficacy, possible degradation of phospholipids and compounds to be encapsulated, elimination of large molecules, metal pollution from probe tip, and presence of MLV along with SUV.
There are two sonication techniques

a) Probe sonication. The tip of a sonicator is directly engrossed into the liposome dispersion. The energy input into lipid dispersion is very high in this method. The coupling of energy at the tip results in local hotness; therefore, the vessel must be engrossed into a water/ice bath. Throughout the sonication up to 1 h, more than 5% of the lipids can be de-esterified. Also, with the probe sonicator, titanium will slough off and pollute the solution.

b) Bath sonication. The liposome dispersion in a cylinder is placed into a bath sonicator. Controlling the temperature of the lipid dispersion is usually easier in this method, in contrast to sonication by dispersal directly using the tip. The material being sonicated can be protected in a sterile vessel, dissimilar the probe units, or under an inert atmosphere.

French Pressure Cell: Extrusion

French pressure cell involves the extrusion of MLV through a small orifice. An important feature of the French press vesicle method is that the proteins do not seem to be significantly pretentious during the procedure as they are in sonication. An interesting comment is that French press vesicle appears to recall entrapped solutes significantly longer than SUVs do, produced by sonication or detergent removal.

The method involves gentle handling of unstable materials. The method has several advantages over sonication method. The resulting liposomes are rather larger than sonicated SUVs. The drawbacks of the method are that the high temperature is difficult to attain, and the working volumes are comparatively small (about 50 mL as the maximum).

Freeze-Thawed Liposomes

SUVs are rapidly frozen and thawed slowly. The short-lived sonication disperses aggregated materials to LUV. The creation of unilamellar vesicles is as a result of the fusion of SUV throughout the processes of freezing and thawing. This type of synthesis is strongly inhibited by increasing the phospholipid concentration and by increasing the ionic strength of the medium. The encapsulation efficacies from 20% to 30% were obtained.

Solvent Dispersion Method

Ether Injection (Solvent Vaporization)

A solution of lipids dissolved in diethyl ether or ether-methanol mixture is gradually injected to an aqueous solution of the material to be encapsulated at 55°C to 65°C or under reduced
pressure. The consequent removal of ether under vacuum leads to the creation of liposomes. The main disadvantages of the technique are that the population is heterogeneous (70 to 200 nm) and the exposure of compounds to be encapsulated to organic solvents at high temperature.

**Ethanol Injection**

A lipid solution of ethanol is rapidly injected to a huge excess of buffer. The MLVs are at once formed. The disadvantages of the method are that the population is heterogeneous (30 to 110 nm), liposomes are very dilute, the removal all ethanol is difficult because it forms into azeotrope with water, and the probability of the various biologically active macromolecules to inactivate in the presence of even low amounts of ethanol is high.

**Reverse Phase Evaporation Method**

This method provided a progress in liposome technology, since it allowed for the first time the preparation of liposomes with a high aqueous space-to-lipid ratio and a capability to entrap a large percentage of the aqueous material presented. Reverse-phase evaporation is based on the creation of inverted micelles. These inverted micelles are shaped upon sonication of a mixture of a buffered aqueous phase, which contains the water-soluble molecules to be encapsulated into the liposomes and an organic phase in which the amphiphilic molecules are solubilized. The slow elimination of the organic solvent leads to the conversion of these inverted micelles into viscous state and gel form. At a critical point in this process, the gel state collapses, and some of the inverted micelles were disturbed. The excess of phospholipids in the environment donates to the formation of a complete bilayer around the residual micelles, which results in the creation of liposomes. Liposomes made by reverse phase evaporation method can be made from numerous lipid formulations and have aqueous volume-to-lipid ratios that are four times higher than hand-shaken liposomes or multilamellar liposomes.

Briefly, first, the water-in-oil emulsion is shaped by brief sonication of a two-phase system, containing phospholipids in organic solvent such as isopropyl ether or diethyl ether or a mixture of isopropyl ether and chloroform with aqueous buffer. The organic solvents are detached under reduced pressure, resulting in the creation of a viscous gel. The liposomes are shaped when residual solvent is detached during continued rotary evaporation under reduced pressure. With this method, high encapsulation efficiency up to 65% can be obtained in a medium of low ionic strength for example 0.01 M NaCl. The method has been used to
encapsulate small, large, and macromolecules. The main drawback of the technique is the contact of the materials to be encapsulated to organic solvents and to brief periods of sonication. These conditions may possibly result in the breakage of DNA strands or the denaturation of some proteins. Modified reverse phase evaporation method was presented by Handa et al., and the main benefit of the method is that the liposomes had high encapsulation efficiency (about 80%).

**Detergent Removal Method (Removal of Non-Encapsulated Material)**

**Dialysis**

The detergents at their critical micelle concentrations (CMC) have been used to solubilize lipids. As the detergent is detached, the micelles become increasingly better-off in phospholipid and lastly combine to form LUVs. The detergents were removed by dialysis. A commercial device called LipoPrep (Diachema AG, Switzerland), which is a version of dialysis system, is obtainable for the elimination of detergents. The dialysis can be performed in dialysis bags engrossed in large detergent free buffers (equilibrium dialysis).

**Detergent (Cholate, Alkyl Glycoside, Triton X-100) Removal Of Mixed Micelles (Absorption)**

Detergent absorption is attained by shaking mixed micelle solution with beaded organic polystyrene adsorbers such as XAD-2 beads (SERVA Electrophoresis GmbH, Heidelberg, Germany) and Bio-beads SM2 (Bio-Rad Laboratories, Inc., Hercules, USA). The great benefit of using detergent adsorbers is that they can eliminate detergents with a very low CMC, which are not entirely depleted.

**Gel-Permeation Chromatography**

In this method, the detergent is depleted by size special chromatography. Sephadex G-50, Sephadex G-1 00 (Sigma-Aldrich, MO, USA), Sepharose 2B-6B, and Sephacryl S200-S1000 (General Electric Company, Tehran, Iran) can be used for gel filtration. The liposomes do not penetrate into the pores of the beads packed in a column. They percolate through the inter-bead spaces. At slow flow rates, the separation of liposomes from detergent monomers is very good. The swollen polysaccharide beads adsorb substantial amounts of amphiphilic lipids; therefore, pre-treatment is necessary. The pre-treatment is done by pre-saturation of the gel filtration column by lipids using empty liposome suspensions.
**Drug Loading In Liposomes**

Drug loading can be attained either passively (i.e., the drug is encapsulated during liposome formation) or actively (i.e., after liposome formation). Hydrophobic drugs, for example amphotericin B taxol or annamycin, can be directly combined into liposomes during vesicle formation, and the amount of uptake and retention is governed by drug-lipid interactions. Trapping effectiveness of 100% is often achievable, but this is dependent on the solubility of the drug in the liposome membrane. Passive encapsulation of water-soluble drugs depends on the ability of liposomes to trap aqueous buffer containing a dissolved drug during vesicle formation. Trapping effectiveness (generally <30%) is limited by the trapped volume delimited in the liposomes and drug solubility. On the other hand, water-soluble drugs that have protonizable amine functions can be actively entrapped by employing pH gradients, which can result in trapping effectiveness approaching 100%.

**Freeze-Protectant For Liposomes (Lyophilization)**

Natural excerpts are usually degraded because of oxidation and other chemical reactions before they are delivered to the target site. Freeze-drying has been a standard practice employed to the production of many pharmaceutical products. The overwhelming majority of these products are lyophilized from simple aqueous solutions. Classically, water is the only solvent that must be detached from the solution using the freeze-drying process, but there are still many examples where pharmaceutical products are manufactured via a process that requires freeze-drying from organic co-solvent systems.

Freeze-drying (lyophilization) involves the removal of water from products in the frozen state at tremendously low pressures. The process is normally used to dry products that are thermo-labile and would be demolished by heat-drying. The technique has too much potential as a method to solve long-term stability difficulties with admiration to liposomal stability. Studies showed that leakage of entrapped materials may take place during the process of freeze-drying and on reconstitution. Newly, it was shown that liposomes when freeze-dried in the presence of adequate amounts of trehalose (a carbohydrate commonly found at high concentrations in organism) retained as much as 100% of their original substances. It shows that trehalose is an excellent cryoprotectant (freeze-protectant) for liposomes. Freeze-driers range in size from small laboratory models to large industrial units available from pharmaceutical equipment suppliers.
Mechanism of Transportation Through Liposome

The limitations and benefits of liposome drug carriers lie critically on the interaction of liposomes with cells and their destiny in vivo after administration. In vivo and in vitro studies of the contacts with cells have shown that the main interaction of liposomes with cells is either simple adsorption (by specific interactions with cell-surface components, electrostatic forces, or by non-specific weak hydrophobic) or following endocytosis (by phagocytic cells of the reticuloendothelial system, for example macrophages and neutrophils).

Fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal content into the cytoplasm, is much rare. The fourth possible interaction is the exchange of bilayer components, for instance cholesterol, lipids, and membrane-bound molecules with components of cell membranes. It is often difficult to determine what mechanism is functioning, and more than one may function at the same time.

Techniques of Liposome Preparation

In different preparation procedures a general pattern can be discerned

1. The lipid must be hydrated, then
2. liposomes have to be sized, and finally
3. Non-encapsulated drug has to be removed.

In some preparation schemes the hydration and sizing steps are combined.

Sometimes all drugs are liposomes associated and no free drug can be found after stage 2 then stage 3 is lacking.

1) Hydration stage

a) Mechanical Methods: MLVs were traditionally produced by hydrating thin lipids films deposited from an organic solution on a glass wall by shaking at temperatures above the phase transition temperature of the phospholipid with the highest Tc. The wide size distributions of the produced liposome dispersions were usually narrowed down by (low) pressure extrusion or ultra-sonication.

b) Methods based on replacement of organic solvent by aqueous media: The lipid constituents are first dissolved in an organic solvent which is subsequently brought in contact with an aqueous phase. The organic solvent is removed later. During the removal of the
organic phase, liposome are formed. Their characteristics (size, organisation of bilayers) depend on the protocol used. If the organic solvent with the dissolved lipids is not miscible with the aqueous phase (ether, chloroform, freons), then the intermediate stage is an emulsion (immiscible solvent). Other organic solvents containing the dissolved lipid(s) can be mixed homogenously with the aqueous phase (ethanol) in the first stage.

Then liposomes formation occurs when the organic solvent concentration drops below a certain critical value (misciblesolvents). The contents of residual organic solvent that is exceptable in the finished product depends on the solvent in question and the route of administration. Apart from evaporation, techniques similar to those used to remove nonencapsulated material can be selected: gel permeation, ultracentrifugation, dialysis. Organic solvent may contain impurities with a high affinity for bilayers; they may be enriched in the bilayer and cause safety or stability problems. Diethyl ether for instance, can be contaminated with peroxide that accumulates in the bilayer. Freshly (from bisulphite) distilled ether should therefore be used.

c) Methods based on detergent removal: (Phospho)lipids, lipophilic compounds and amphiphatic proteins can be solubilized by detergents forming mixed micells. Upon removal of the detergent, vesicle formation can occur. This technique is well established for preparation of reconstituted virus envelopes or reconstituted tumor membrane material. Schreier and coworkers described a two step strategy for insertion of proteins into the outer layer of liposomes. First liposomes were formed by detergent dialysis method and subsequently proteins were inserted by partial resolubalization of the membrane by the detergent (deoxycolate) in the presence of protein.

d) Method based on size transformation and fusion: Sonication of phospholipids below their phase transition temperature (Tc) results in vesicles with defects in the bilayers. Heating the dispersion to Tc eliminates these structural defects and causes fusion resulting in large unilamellar liposomes with a wide size distribution. Main disadvantage of this process is the limited number of bilayer composition that reacts and the poor reproducibility of the particle size distribution of the liposome dispersion that is formed.

2) Sizing stage
There are two approaches, one without a special sizing step [A] and One with a special sizing step [B]
[A] In liposome formation process, circumstances are selected and controlled in such a way that particle size distributions with an acceptable width are produced. High shear homogenization produces a size distribution which depends on operational pressure.

[B] For small dispersion volumes, the liposome dispersion can be fractionated by centrifugation as liposome density usually differs from the density of the medium. Gel permeation chromatography has also been used for subdividing wide particle size distribution. On an analytical or semipreparative scale, the selection of the pore size of the chromatographic material provides an opportunity to manipulate the size class resolution within certain limits.

3) Removal of non-encapsulated material
Many lipophilic drugs exhibit a high affinity to the bilayer and are completely liposome associated. However, for other compounds, the encapsulation efficiency is less than 100 percent. The nonencapsulated fraction of the active compound can cause unacceptable side effects or physical instability.

For removal of nonencapsulated material, the following techniques are used:

a) dialysis and ultracentrifugation, b) Gel permeation chromatography, c) Ion exchange reactions.

Stability of Liposomes
Liposomes face a number of chemical and physical destabilisation processes. So liposomes stability is an important consideration while studying liposomes. This aspect of liposomes stability have two aspects physical and chemical stability.

Physical Stability
Physical processes that effect shelf life include loss of liposome associated drug and changes in size, aggregation and fusion. Aggregation is the formation of larger units of liposome material, these units are still composed of individual liposomes. In principle, this process is reversible, e.g., by applying mild shear forces, or by changing the temperature or by binding metal ions that initially induced aggregation. Fusion indicates that new colloidal structure were formed. As fusion is an irreversible process, the original liposomes can never be retrived. Drug molecules can leak from liposomes. The leakage rate strongly depends on the bilayer composition and the physiochemical nature of the drug. Bilayers in the gel state or
those containing substantial (molar) fractions of cholesterol tend to lose associated drug only slowly; liquid state bilayers are more prone to drug loss and are less stable during storage. Bilayer permeability is not necessarily a constant parameter. Change in bilayer permeability can occur as a result of chemical degradation process, such as formation of lypo-PC and FA.

**Chemical Stability**

**a) Hydrolysis of the ester bonds**

Phosphatidylcholine possesses for ester bonds. The two acyl ester bonds are most liable to hydrolysis. The glycerophosphate and phosphocholine ester bonds are more stable. The 1-acyl-lysophosphatidylcholine (LPC) and 2acyl LPC are both formed at comparable rates.

**b) Lipid peroxidation of phospholipids**

The polyunsaturated acyl chains of phospholipids are sensitive to oxidation via free radical reactions. Cyclic peroxides, hydroperoxides, malonilaldehyde, alkanes are the major degradation products. Low oxygen pressure, absence of heavy metals, addition of anti-oxidants, complexing agents(EDTA,etc), quenchers(beta-carotene) of the photo-oxidatio reactions improve resistance against lipid peroxidation.

**PHYSICAL TARGETING**

In this type of targeting some characteristics of environment changes like pH, temperature, light intensity, electric field, ionic strength small and even specific stimuli like glucose concentration are used to localize the drug carrier to predetermined site. This approach was found exceptional for tumor targeting as well as cytosolic delivery of entrapped drug or genetic material.

<table>
<thead>
<tr>
<th>Physical Targeting</th>
<th>Formulation System</th>
<th>Mechanism For Drug Delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat</td>
<td>Liposome</td>
<td>Change in Permeability</td>
</tr>
<tr>
<td>Magnetic Modulation</td>
<td>Magnetically Responsive Microspheres</td>
<td>Magnetic Field can retard fluid Flow of particles</td>
</tr>
<tr>
<td></td>
<td>Containing Iron oxide</td>
<td></td>
</tr>
<tr>
<td>Ultrasound</td>
<td>Polymers</td>
<td>Change in Permeability</td>
</tr>
<tr>
<td>Electrical Pulse</td>
<td>Gels</td>
<td>Change in Permeability</td>
</tr>
<tr>
<td>Light</td>
<td>Photo responsive Hydro gels Containing azo Derivatives</td>
<td>Change in Diffusion Channels, Activated by Specific Wavelength</td>
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LIPOSOMES

Liposomes are simple microscopic vesicles in which an aqueous volume is entirely composed by membrane of lipid molecule various amphiphelic molecules have been used to form liposomes. The drug molecules can either be encapsulated in aqueous space or intercalated into the lipid bilayers. The extent of location of drug will depend upon its physico-chemical characteristics and composition of lipids.

To improve liposome stability and enhance their circulation times in the blood, sterically-stabilized liposomes were introduced. The hydrophilic polymer, polyethylene glycol (PEG), has been shown to be the optimal choice for obtaining sterically-stabilized liposomes. The establishment of a steric barrier improves the efficacy of encapsulated agents by reducing \textit{in vivo} opsonization with serum components, and the rapid recognition and uptake by the RES. This not only reduces the elimination of drugs by prolonging blood circulation and providing accumulation at pathological sites, but also attenuates side effects. Steric stabilization strongly influences the pharmacokinetics of liposomes, with reported half-lives varying from 2 to 24 h in rodents (mice and rats) and as high as 45 h in humans, depending on the particle size and the characteristics of the coating. While coating liposomes with PEG results in prolonged circulation times, there can be an offsetting reduction in the ability to interact with the intended targets.

Ligand-targeted liposomes offer a vast potential for site-specific delivery of drugs to designated cell types or organs \textit{in vivo}, which selectively express or over-express specific ligands (e.g., receptors or cell adhesion molecules) at the site of disease. Many types of ligands are available, such as antibodies, peptides/proteins and carbohydrates. The coupling of antibodies, particularly monoclonal antibodies, to create immune-liposomes represents one of the more versatile ligands that can be affixed to liposome surface. One of the advantages of using monoclonal antibodies is their stability and higher binding avidity because of the presence of two binding sites on the molecule. Since lipid assemblies are usually dynamic structures, surface-coupled ligands have a high motional freedom to position themselves for optimal substrate-interactions. The limited \textit{in vivo} performance of immuno-liposomes, due to poor pharmacokinetics and immunogenicity, has been a major hurdle to achieving their potential as effective site-specific drug carriers. Therefore, newer generation of liposomes have utilized a combination of the above design platforms to further improve liposomal targeting and associated drug delivery. For example, integrating target-specific binding of
immuno-liposomes with the steric stabilization of PEG (thereby creating long-circulating immuno-liposomes) has significantly improved the pharmacokinetics of immuno-liposomes. Overall as a drug delivery platform, liposomes offer a dynamic and adaptable technology for enhancing the systemic efficacy of therapeutics in various diseases.

Fusogenic Liposomes and Antibody-Mediated Liposomes in Cancer Therapy

It has been infrequently well-known that a powerful anticancer drug, especially one that targets the cytoplasm or cell nucleus, does not work due to the low permeability across a plasma membrane, degradation by lysosomal enzymes through an endocytosis-dependent pathway, and other reasons. Thus, much attention on the use of drug delivery systems is focused on overcoming these problems, ultimately leading to the induction of maximal ability of anti-cancer drug. In this respect, a new model for cancer therapy using a novel drug delivery system, fusogenic liposome, was developed.

Fusogenic liposomes are poised of the ultraviolet-inactivated Sendai virus and conventional liposomes. Fusogenic liposomes effectively and directly deliver their encapsulated contents into the cytoplasm using a fusion mechanism of the Sendai virus, whereas conventional liposomes are taken up by endocytosis by phagocytic cells of the reticuloendothelial system, for example macrophages and neutrophils. Thus, fusogenic liposome is a good candidate as a vehicle to deliver drugs into the cytoplasm in an endocytosis-independent manner.

Liposomal drug delivery systems provide steady formulation, provide better pharmacokinetics, and make a degree of ‘passive’ or ‘physiological’ targeting to tumor tissue available. However, these transporters do not directly target tumor cells. The design modifications that protect liposomes from unwanted interactions with plasma proteins and cell membranes which differed them with reactive carriers, for example cationic liposomes, also prevent interactions with tumor cells. As an alternative, after extravasation into tumor tissue, liposomes remain within tumor stroma as a drug-loaded depot. Liposomes ultimately become subject to enzymatic degradation and/or phagocytic attack, leading to release of drug for subsequent diffusion to tumor cells. The next generation of drug carriers under development features directs molecular targeting of cancer cells via antibody-mediated or other ligand-mediated interactions.
Applications of Liposomes in Medicine and Pharmacology

Applications of liposomes in medicine and pharmacology can be divided into diagnostic and therapeutic applications of liposomes containing various markers or drugs, and their use as a tool, a model, or reagent in the basic studies of cell interactions, recognition processes, and mode of action of certain substances.

Unfortunately, many drugs have a very narrow therapeutic window, meaning that the therapeutic concentration is not much lower than the toxic one. In several cases, the toxicity can be reduced or the efficacy can be enhanced by the use of a suitable drug carrier which alters the temporal and spatial delivery of the drug, i.e., its bio-distribution and pharmacokinetics. It is clear from many pre-clinical and clinical studies that drugs, for instance antitumor drugs, parcelled in liposome demonstration reduced toxicities, while retentive enhanced efficacy.

Advances in liposome design are leading to new applications for the delivery of new biotechnology products, for example antisense oligonucleotides, cloned genes, and recombinant proteins. A vast literature define the viability of formulating wide range of conservative drugs in liposomes, frequently resultant in improved therapeutic activity and/or reduced toxicity compared with the free drug. As a whole, changed pharmacokinetics for liposomal drugs can lead to improved drug bioavailability to particular target cells that live in the circulation, or more prominently, to extravascular disease sites, for example, tumors. Recent improvements include liposomal formulations of all-trans-retinoic acid and daunorubicin, which has received Food and Drug Administration consent as a first-line treatment of AIDS-related advanced Kaposi's sarcoma. Distinguished examples are vincristine, doxorubicin, and amphotericin B.

The benefits of drug load in liposomes, which can be applied as (colloidal) solution, aerosol, or in (semi) solid forms, such as creams and gels, can be summarized into seven categories (Table 2).
Benefits of drug load in liposomes

<table>
<thead>
<tr>
<th>Benefits of Drug Load In Liposome</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Improved solubility of lipophilic and amphiphilic drugs</td>
<td>Amphotericin B, porphyrins, minoxidil, some peptides, and anthracyclines, respectively; hydrophilic drugs, such as anticancer agent doxorubicin or acyclovir</td>
</tr>
<tr>
<td>2. Passive targeting to the cells of the immune system, especially cells of the mononuclear phagocytic system</td>
<td>Antimonials, amphotericin B, porphyrins, vaccines, immunomodulators</td>
</tr>
<tr>
<td>3. Sustained release system of systemically or locally administered liposomes</td>
<td>Doxorubicin, cytosine arabinoside, cortisones, biological proteins or peptides such as vasopressin</td>
</tr>
<tr>
<td>4. Site-avoidance mechanism</td>
<td>Doxorubicin and amphotericin B</td>
</tr>
<tr>
<td>5. Site-specific targeting</td>
<td>Anti-inflammatory drugs, anti-cancer, anti-infection</td>
</tr>
<tr>
<td>6. Improved transfer of hydrophilic, charged molecules</td>
<td>Antibiotics, chelators, plasmids, and genes</td>
</tr>
<tr>
<td>7. Improved penetration into tissues</td>
<td>Corticosteroids, anesthetics, and insulin</td>
</tr>
</tbody>
</table>

LIPOSOMES IN PARASITIC DISEASES AND INFECTIONS

From the time when conventional liposomes are digested by phagocytic cells in the body after intravenous management, they are ideal vehicles for the targeting drug molecules into these macrophages. The best known instances of this ‘Trojan horse-like’ mechanism are several parasitic diseases which normally exist in the cell of MPS. They comprise leishmaniasis and several fungal infections.

Leishmaniasis is a parasitic infection of macrophages which affects over 100 million people in tropical regions and is often deadly. The effectual dose of drugs, mostly different antimonials, is not much lower than the toxic one. Liposomes accumulate in the very same cell population which is infected, and so an ideal drug delivery vehicle was proposed. Certainly, the therapeutic index was increased in rodents as much as several hundred times upon administration of the drug in various liposomes. Unexpectedly, and unfortunately, there was not much interest to scale up the formulations and clinically approve them after several very encouraging studies dating back to 1978. Only now, there are several continuing studies with various anti-parasitic liposome formulations in humans. These formulations use mostly ionosphere amphotericin B and are transplanted from very successful and prolific area of liposome formulations in antifungal therapy.

The best results reported so far in human therapy are probably liposomes as carriers for amphotericin B in antifungal therapies. This is the drug of choice in dispersed fungal
infections which often in parallel work together with chemotherapy, immune system, or AIDS, and is frequently fatal. Unfortunately, the drug itself is very toxic and its dosage is limited due to its ionosphere and neurotoxicity. These toxicities are normally related with the size of the drug molecule or its complex. Obviously, liposome encapsulation inhibits the accumulation of drug in these organs and radically reduces toxicity. Furthermore, often, the fungus exists in the cells of the mononuclear phagocytic system; therefore, the encapsulation results in reduced toxicity and passive targeting. These benefits, however, can be associated with any colloidal drug carrier. Certainly, similar improvements in therapy were observed with stable mixed micellar formulations and micro-emulsions. Additionally, it seems that many of the early liposomal preparations were in actual fact liquid crystalline colloidal particles rather than self-closed MLV. Since the lives of the first terminally ill patients (who did not rely to all the conventional therapies) were saved, many patients were very effectively treated with diverse of amphotericin B formulations.

Comparable methods can be achieved in antiviral and antibacterial therapies. Most of the antibiotics, however, are orally available; liposome encapsulation can be considered only in the case of very potent and toxic ones which are administered parenterally. The preparation of antibiotic-loaded liposomes at sensibly high drug-to-lipid ratios may not be easy because of the interactions of these molecules with bilayers and high densities of their aqueous solutions which often force liposomes to float as a creamy layer on the top of the tube. Several other ways, for instance, topical or pulmonary (by inhalation) administration are being considered also. Liposome-encapsulated antivirals (for example ribavirin, azidothymidine, or acyclovir) have also shown to reduce toxicity; currently, more detailed experiments are being performed in relation to their efficacy.

**LIPOSOMES IN ANTICANCER THERAPY**

Numerous different liposome formulations of numerous anticancer agents were shown to be less toxic than the free drug. Anthracyclines are drugs which stop the growth of dividing cells by intercalating into the DNA and, thus, kill mainly rapidly dividing cells. These cells are not only in tumors but are also in hair, gastrointestinal mucosa, and blood cells; therefore, this class of drug is very toxic. The most used and studied is Adriamycin (commercial name for doxorubicin HCl; Ben Venue Laboratories, Bedford, Ohio). In addition to the above-mentioned acute toxicities, its dosage is limited by its increasing cardio toxicity. Numerous diverse formulations were tried. In most cases, the toxicity was reduced to about 50%. These
include both acute and chronic toxicities because liposome encapsulation reduces the delivery of the drug molecules towards those tissues. For the same reason, the efficiency was in many cases compromised due to the reduced bioavailability of the drug, especially if the tumor was not phagocytic or located in the organs of mononuclear phagocytic system. In some cases, such as systemic lymphoma, the effect of liposome encapsulation showed enhanced efficacy due to the continued release effect, i.e., longer presence of therapeutic concentrations in the circulation, while in several other cases, the sequestration of the drug into tissues of mononuclear phagocytic system actually reduced its efficacy.

Applications in man showed, in general, reduced toxicity and better tolerability of administration with not too encouraging efficacy. Several different formulations are in different phases of clinical studies and show mixed results.

CONCLUSIONS
Liposomes have been used in a broad range of pharmaceutical applications. Liposomes are showing particular promise as intracellular delivery systems for anti-sense molecules, ribosomes, proteins/peptides, and DNA. Liposomes with enhanced drug delivery to disease locations, by ability of long circulation residence times, are now achieving clinical acceptance. Also, liposomes promote targeting of particular diseased cells within the disease site. Finally, liposomal drugs exhibit reduced toxicities and retain enhanced efficacy compared with free complements. Only time will tell which of the above applications and speculations will prove to be successful. However, based on the pharmaceutical applications and available products, we can say that liposomes have definitely established their position in modern delivery systems.

REFERENCES