INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACY AND CHEMISTRY

Available online at www.ijrpc.com

Review Article

LIPOSOMES – THE FUTURE OF FORMULATIONS

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ABSTRACT

Liposomes were discovered in the early 1960's by Bangham and collegues. It took many years for the development of liposomes into potential and effective drug delivery systems. Initially they were used to study biomembrane behavior but later on developed into a drug delivery system for targeting specific sites of action like the tumor targeting, gene and antisense therapy, genetic vaccination, immunomodulation, various infections etc. Liposomes have an extensive application ranging from medicine to textile industry, cosmetics, chemistry, etc. Hence, being a very potential drug delivery system that has extensive applications in the future, it is very useful to study different aspects of liposomes. This review article summarizes some important methods of preparation, applications and commercial issues related to liposomes.

Keywords: Liposomes, plasmalogens, gene therapy, controlling drug delivery.

INTRODUCTION

Liposome research is gaining importance in biological, pharmaceutical and medical research because liposomes seem to be the most effective carriers for the introduction of all kinds of agents into cells. Liposomes, which are lipid microspheres, have a wide spectrum of applications ranging from use in basic research in biophysics to various practical applications like cosmetics and pharmaceuticals, production of ultrafine particles, and many more. Liposomes are used as model systems for studying biomembranes and their properties, such as permeability, as a function of chemical composition of the membrane. In biochemistry, they enable the scientists to reconstitute and investigate membrane proteins in a well-defined environment which closely mimics the natural one. The most important use of liposomes is expected to be in pharmacology, medicine and biotechnology, where they serve as vehicles for controlling the delivery of entrapped drugs, genetic material, enzymes and other macromolecules. Liposomes have also attracted the attention of the food and cosmetic industries, where it is possible to form insoluble molecules in water-based liposomes, pastes and ointments.

DEFINITION

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

Hence, microscopic, fat-containing, bubble created in the laboratory which is used to deliver drugs through the bloodstream are called lipososmes. They are lipid nanoparticles.

Liposomes were first produced in England in 1961 by Alec D. Bangham, who was studying phospholipids and blood clotting. It was found that phospholipids combined with water immediately formed a sphere because one end of each molecule is water soluble, while the opposite end is water insoluble. Water-soluble medications added to the water were trapped inside the aggregation of the hydrophobic ends; fat-soluble medications were incorporated into the phospholipid layer.

In some cases liposomes attach to cellular membranes and appear to fuse with them, releasing their contents into the cell. Sometimes they are taken up by the cell, and their phospholipids are incorporated into the cell membrane while the drug trapped inside is released. In the case of phagocytic cells, the liposomes are taken up, the phospholipid walls are acted upon by organelles called lysosomes, and the medication is released. Liposomal delivery systems are still largely experimental; the precise mechanisms of their action in the body are under study, as are ways in which to target them to specific diseased tissues.

CLASSIFICATION

Liposomes are often distinguished according to their number of lamellae and size. Small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and large multilamellar vesicles (MLV) or multivesicular vesicles (MVV) are differentiated. SUVs show a diameter of 20 to approximately 100 nm. LUVs, MLVs, and MVVs range in size from a few hundred nanometers to several microns. The thickness of the membrane (phospholipid bilayer) measures approximately 5 to 6 nm.

Large liposomes form spontaneously when phospholipids are dispersed in water above their phase transition temperature. The preparation of SUVs starts usually with MLVs, which then are transformed into small vesicles using an appropriate manufacturing technique, e.g. high-pressure homogenization.

Here is a summary of classification of liposomes:

TYPES	SPECIFICATIONS
Based on structural parameters	
MLV	Multilamellar vesicles - >0.5μm
OLV	Oligolamellar vesicles – 0.1 - 1μm
UV	Unilamellar vesicles (all size ranges)
SUV	Small unilamellar vesicles – 20 – 100nm
MUV	Medium sized unilamellar vesicles
LUV	Large unilamellar vesicles - >100nm
GUV	Giant unilamellar vesicles - >1μm
MV	Multivesicular vesicles - >1μm
Based on method of preparation	
REV	Single or oligolamellar vesicles made by reverse phase evaporation method
MLV – REV	Multilamellar vesicles made by reverse phase evaporation method
SPLV	Stable plurilamellar vesicles
FATMLV	Frozen and thawed MLV
VET	Vesicles prepared by extrusion technique
DRV	Dehydration - rehydration method
Based on composition and applications	
Conventional Liposomes (CL)	Neutral or negatively charged phospholipids and cholesterol
Fusogenic liposomes (RSVE)	Reconstituted Sendal virus envelope
pH sensitive liposomes	Phospholipids like PEs(1,2-diacyl-sn glycero-3-phosphorylethanolamine, phosphatidylethanolamine) and DOPEs dioleoylphosphatidylethanolamine cholesterol / tetradecane with either CHEMS or OA
Cationic liposomes	Positively charged cationic lipids with DOPE used to transport DNA
Long circulatory (stealth) lipososmes (LCL)	Neutral high Tc, Chol and 5 – 10% of PEG – DSPE or GM1
Immuno liposomes	CL or LCL with attached monoclonal antibodies or recognition sequences

FORMATION OF LIPOSOMES

Liposomes are made of phospholipid molecues that have a hydrophobic tail composed of 2 fatty acid chains (10 – 24 carbon atoms) and a hydrophilic polar end made of phosphoric acid that may or may not be esterified with alcoholic functional groups. The amphiphilic nature of these molecules is the reason for their characteristic orientation and self organization into supramolecular structures.

The most commonly used natural phospholipid is the PC (phosphatidyl choline). The molecules are not soluble in aqueous medium but are dispersible it. When in aqueous medium, they align themselves closely in planar bilayer sheets to minimize the unfavorable interactions between aqueous and the non aqueous media. When the sheets fold over themselves these interactions are completely eliminated. Thus they separate the two phases with the polar portion in contact with water and the non polar portion in contact with the lipophilic environment.

The orientation is mostly bilayered but not micellar due to the amphiphilic nature unlike in other amphiphilic molecules like soaps. This is because of the double fatty acid chain that results in a tubular structure making it suitable for planar arrangement.

Depending on the environment, homogenous smectic phases of parallel layers or heterogenous dispersion of multilamellar or singular walled lipsosmes are observed. At lower water contents and high temperatures other crystalline phases like hexagonal, cubic or ribbon occur. There may also be transitions between the gel and fluid phase within narrow temperature ranges.

During the process of forming vesicles, the lipid layers are first hydrated and then swell. When agitated, they detach and form large multilamellar vesicles (MLV). On further application of energy, there may be changes in the morphology and shape. For example, when sonic energy is applied, SUVs are seen and when mechanical energy is applied, extrusion occurs to give LUVs.

The factors for bilayer formation are:

1. Large free energy differences between aqueous and hydrophobic phases.
2. The hydrophobic nteractions and the amphiphilic nature of phospholipids
3. Supramolecular self-assemblage through specific molecular geometry

Examples of some of the phospholipids used are as follows:

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

Synthetic phospholipids

E.g.: for saturated phospholipids are:

1. Dipalmitoyl phosphatidyl choline (DPPC)
2. Distearoyl phosphatidyl choline (DSPC)
3. Dipalmitoyl phosphatidyl ethanolamine (DPPE)
4. Dipalmitoyl phosphatidyl serine (DPPS)
5. Dipalmitoyl phosphatidic acid (DPPA)
6. Dipalmitoyl phosphatidyl glycerol (DPPG)

E.g.: for unsaturated phospholipids

1. Dioleoyl phosphatidyl choline (DOPC)
2. Dioleoyl phosphatidyl glycerol (DOPG)
3. Polymeric materials
4. Synthetic phospholipids with diactylenic group in the hydrocarbon chain polymerizes when exposed to U.V, leading to formation of polymerized liposomes having significantly higher permeability barriers to entrapped aqueous drugs.
5. E.g.: for other Polymerisable lipids are – lipids containing conjugated diene, Methacrylate

Cationic lipids

E.g.: DODAB/C – Dioctadecyl dimethyl ammonium bromide or chloride

DOTAP – Dioleoyl propyl trimethyl ammonium chloride – this is an analogue of DOTAP and various others including various analogues of DOTMA and cationic derivatives of cholesterol

Other Substances

Variety of other lipids of surfactants are used to form liposomes

1. Many single chain surfactants can form liposomes on mixing with cholesterol

2. Non ionic lipids

A variety of Polyglycerol and Polyethoxylated mono and dialkyl amphiphiles used mainly in cosmetic preparations:

1. Single and double chain lipids having fluoro carbon chains can form very stable liposomes
2. Sterylamine and Dicetyl phosphate etc are incorporated into liposomes so as to impart either a negative or positive surface charge to these structures

Use of cholesterol for rigidity

Chlesterol does not form bilayers itself but can incorporate into the phospholipid layers. When incorporated, it acts as fluidity buffer suppressing the tilts and shift in membrane structure at the transition phase.

The concentrations used are very high in the range of 1:1 or 2:1 of cholesterol to PC. At 1:1 ratio, space filling by cholesterol occurs which shows more packing with rows of cholesterol molecules alternating with rows of phospholipids such that they interact closely.

METHODS OF LIPOSOME FORMATION AND DRUG LOADING

There are many ways of preparing lipososmes as listed below. Some of the important methods have been explained.

1. Hydration of lipids in presence of solvent
2. Ultrasonication
3. French Pressure cell
4. Solvent injection method

a) Ether injection method
b) Ethanol injection

5. Detergent removal

Detergent can be removed by

a) Dialysis
b) Coloumn chromatography
c) Bio-beads

6. Reverse phase evaporation technique
7. High pressure extrusion
8. Miscellaneous methods

a) Slow swelling in Non electrolyte solution
b) Removal of Chaotropic ion
c) Freeze-Thawing

Convectional method

In the procedure; the phospholipids are dissolved in an organic solvent (usually a chloroform / methanol mixture) and deposited from the solvents as a thin film on the wall of a round bottom flask by use of rotary evaporation under reduced pressure. MLVs form spontaneously when an excess volume of aqueous buffer containing the drug is added to the dried lipid film. Drug containing liposomes can be separated from nonsequestered drug by centrifugation of the liposomes or by gel filtration. The time allowed for hydration of the dried film and conditions of agitation are critical in determining the amount of the aqueous buffer (or drug solution) that will be entrapped within the internal compartments of the MLVs.

Sonication method

This method is used in the preparation of SUVs and it involves the subsequent sonication of MLVs prepared by the conventional method either with a bath type or a probe type sonicator under an inert atmosphere, usually nitrogen or argon. The principle of sonication involves the use of pulsed, high frequency sound waves (sonic energy) to agitate a suspension of the MLVs. Such disruption of the MLVs produces SUVs with diameter in the range of 15–50nm. The purpose of sonication, therefore, is to produce a homogenous dispersion of small vesicles with a potential for greater tissue penetration. The commonly used sonicators are of the bath and probe tip type. The major drawbacks in the preparation of liposomes by sonication include oxidation of unsaturated bonds in the fatty acid chains of phospholipids and hydrolysis to lysophospholipids and free fatty acids. Another drawback is the denaturation or inactivation of some thermolabile substances (e.g., DNA, certain proteins, etc) to be entrapped.

High-pressure extrusion method

This is another method for converting MLV to SUV suspensions. By this method, suspensions of MLVs prepared by the convectional method are repeatedly passed through filters polycarbonate membranes with very small pore diameter (0.8–1.0μm) under high pressure up to 250psi. By choosing filters with appropriate pore sizes, liposomes of desirable diameters can be produced. The mechanism of action of the high pressure extrusion method appears to be much like peeling an onion. As the MLVs are forced through the small pores, successive layers are peeled off until only one remains. Besides reducing the liposome size, the extrusion method produces liposomes of homogeneous size distributions. A variety of different lipids can be used to form stable

liposomes by this method. Extrusion at low pressures <1Mpa is possible when lipid concentration is low, but the most commonly used pressures are about 5Mpa. A new technique uses 10.5Mpa for better results.

Solubilisation and detergent removal method

This method is used in the preparation of LUVs and it involves the use of detergent (surfactant) for the solubilisation of the lipids. Detergents used include the non-ionic surfactants [e.g., n-octyl-bete-D-glucopyranose (octyl gluside), anionic surfactants (e.g., dodecyl sulphate) and cationic surfactants (e.g., hexadecyltrimethyl ammoniumbromide). The procedure involves the solubilisation of the lipids in an aqueous solution of the detergent and the protein(s) to be encapsulated. The detergent should have a high critical micelle concentration (CMC), so that it is easily removed. The detergent is subsequently removed by dialysis or column chromatography. During detergent removal, LUVs of diameter 0.08–0.2μm are produced. This detergent removal method has been found suitable for the encapsulation of proteins of biomedical importance.

Reverse phase evaporation technique

It consists of a rapid injection of aqueous solution of the drug into an organic solvent, which contains the lipid dissolved with simultaneous bath sonication of the mixture leading to the formation of water droplets in the organic solvent (i.e., a "water-in-oil" emulsion). The resulting emulsion is dried down to a semi solid gel in a rotary evaporator. The next step is to subject the gel to vigorous mechanical agitation to induce a phase reversal from water-in-oil to oil-in-water dispersion (i.e., an aqueous suspension of the vesicles). During the agitation, some of the water droplets collapse to form the external phase while the remaining portion forms the entrapped aqueous volume. Large unilamellar vesicles (diameter 0.1–1μm) are formed in the process. This method has been used to encapsulate both small and macromolecules such as RNA and various enzymes without loss of activity. The expected limitation of this method is the exposure of the material to be encapsulated to organic solvents and mechanical agitation, which can lead to the denaturation of some proteins or breakage of DNA strands. Reports of such limitations are however rare in the literatures.

CHARACTERIZATION OF LIPOSOMES

It is done after the liposomes have been manufactured to ensure their predictable in vitro (sterilization and shelf life) and in vivo (disposition) performances. The characterization is done in three categories: physical, chemical, and biological. The various parameters and the tests used therein are listed below.

Characterization parameters	Analytical methods/Instrumentation
Chemical characterization	
Phospholipid concentration	Lipid phosphorus by Barlettassa/Stewartassa, HPLC
Cholesterol concentration	Cholesterol oxidase assay, HPLC
Drug concentration	Tests according to monograph of each drug
Phospholipid peroxidation	UV absorbance, TBA (for endoperoxidase), iodometric (for hydroperoxidase), and GLC
Phospholipid hydrolysis	HPLC, TLC, fatty acid concentration
Cholesterol auto oxidation	HPLC, TLC
Anti-oxidant degradation	HPLC, TLC
pH	pH meter
Osmolarity	Osmometer
Physical characterization	
Vesicle shape and surface morphology	TEM, freeze fracture electron microscopy
Vesicle size and size distribution Sub micron range Micron range	 Dynamic light scattering, TEM, zeta sizer TEM, photon correlation spectroscopy, Laser light scattering, gel permeation, and gel exclusion, Freeze fracture electron microscopy,
Surface charge	Free flow electrophoresis
Electron surface potential and surface pH	Zeta potential measurements and pH sensitive probes

Lamellarity	Small angle x-rascattering, freeze fracture electron microscopy, P-13NMR
Phase behaviour	Freeze-fracture electron microscopy, DSC
Percent capture / percent of free drug	Mini column centrifugation, gel exclusion, ion-exchange chromatopgraphy, protamine aggregation, radiolabelling
Drug release	Diffusion cell / dialysis
Biological characterization	
Sterility	Aerobic and anaerobic cultures
Progenicity	Rabbit fever response test or Limulus Amebocte Lysate test
Animal toxicity	Monitoring survival rates, histology and pathology

STABILITY OF LIPOSOMES

The stability studies are done in two aspects: in vitro and in vivo. The former covers the aspects before administration of the formulation and also the stability of consecutive lipids. The latter covers the aspects after the administration of the formulation to the biological system.

There is no pre-determined protocol for conducting these studies – neither the accelerated stability studies nor the long term stability studies. The tests are done based on the environmental conditions.

The various tests are conducted in the following categories:

Chemistry, Manufacturing, and Controls

A. Description and Composition
B. Physicochemical Properties
C. Description of Manufacturing Process and Process Controls
D. Control of Excipients: Lipid Components
E. Control of Drug Product: Specifications
F. Stability
G. Changes in Manufacturing

Human pharmacokinetics and Bioavailability

A. Bioanalytical Methods
B. In Vivo Integrity (Stability) Considerations
C. Protein Binding
D. In Vitro Stability
E. Pharmacokinetics and Bioavailability

PHARMACOKINETIC CONSIDERATIONS

Most small molecular chemotherapeutic agents have a large volume of distribution on intravenous (IV) administration of liposomes. The result of this wide distribution is often a narrow therapeutic index due to a high level of toxicity on healthy tissues. Through encapsulation of drugs in liposomes, the volume of distribution is significantly reduced and the concentration of drug at the desired site of action increased. Liposomes are predominantly removed from circulation by phagocyte cells of the reticuloendothelial system (RES), thus accumulating to a large extent in organs like liver and spleen. This biodistribution pattern can be used for passive targeting of diagnostics to these organs. The RES should, therefore, be saturated with empty vesicles when other sites are the drug targets. Information on biodistribution is, therefore, important for drug targeting by liposomes. Liposomes given intravenously usually interact with at least two distinct groups of plasma proteins. These are the plasma high density lipoproteins and the so-called opsonins, which bind to the surface of vesicles and mediate their endocytosis by the mononuclear phagocyte system (macrophages). The rate of liposome clearance from blood circulation will, therefore, depend on the ability of opsonins to bind to the liposome surface. The rate can be manipulated through appropriate selection of liposome characteristics. Clearance from the blood stream is also influenced by vesicle size and surface charges. The longest half-life is obtained when liposomes are relatively small ($\text{diameter} < 0.05\mu\text{m}$) and carry no net surface charge. The pharmacokinetic behavior of liposomes depends on the route of injection such as intraperitoneal, subcutaneous or intramuscular route. Coating the liposome surface with polyethyleneglycol and other hydrophobic part of phospholipids substantially prolongs the half-life of liposomes in the blood.

APPLICATIONS

Liposomes in the sciences:

Discipline	Application
Mathematics	Topology of two-dimensional surfaces in three-dimensional space governed only by bilayer elasticity
Physics	Aggregation behaviour, fractals, soft and high-strength materials
Biophysics	Permeability, phase transitions in two-dimensions, photophysics
Physical Chemistry	Colloid behavior in a system of well-defined physical characteristics, inter and intra-aggregate forces, DLVO
Chemistry	Photochemistry, artificial photosynthesis, catalysis, micro-compartmentalization
Biochemistry	Reconstitution of membrane proteins into artificial membranes
Biology	Model biological membranes, cell function, fusion, recognition
Pharmaceutics	Studies of drug action
Medicine	Drug-delivery and medical diagnostics, gene therapy

Liposomes in the pharmaceutical industry

Liposome Utility	Current Applications	Disease States Treated
Solubilization	Amphotericin B, minoxidil	Fungal infections
Site-Avoidance	Amphotericin B – reduced nephrotoxicity, doxorubicin – decreased cardiotoxicity	Fungal infections, cancer
Sustained-Release	Systemic antineoplastic drugs, hormones, corticosteroids, drug depot in the lungs	Cancer, biotherapeutics
Drug protection	Cytosine arabinoside, interleukins	Cancer, etc.
RES Targeting	Immunomodulators, vaccines, antimalarials, macophage-located diseases	Cancer, MAI, tropical parasites
Specific Targeting	Cells bearing specific antigens	Wide therapeutic applicability
Extravasation	Leaky vasculature of tumours, inflammations, infections	Cancer, bacterial infections
Accumulation	Prostaglandins	Cardiovascular diseases
Enhanced Penetration	Topical vehicles	Dermatology
Drug Depot	Lungs, sub-cutaneous, intra-muscular, ocular	Wide therapeutic applicability

Other Applications:

1. Treatment of Diseases:

Drug containing liposomes have been shown to be effective against diseases in test animals and in some cases in human beings.

2. As Vaccine Carriers:

Liposomes carrying antigens (derived from infectious organisms) particularly viral antigens, malaria antigens and bacterial toxins have been successfully used to produce humoral or cellular immunity in test animals. This indicates that liposomes have potential as vaccine carriers.

3. In Cell Physiology:

Liposomes can be constituted so that cell membranes are functionally expressed (working like those in natural membranes) on the surface of liposomes, thereby providing a model system for studying receptor-membrane interactions.

As Diagnostic Agents:

The intravenous administration of liposomes containing contrast agents such as 99m Tc have been used to visualize certain malignant tissues such as cancerous tissues of breast, rheumatoid factor, etc.

4. **As Model Membranes:**

i) Liposomes have been used successfully to investigate the mechanism involved in the ototoxicity of aminglycoside antibiotics. On the basis of these studies, the following multistep mechanism was proposed:

(1) Interaction of aminoglycoside with phospholipids in the outer plasma membranes.

(2) Aminoglycoside translocation into cell and

(3) Binding of the drug to phosphatidylinositol 4, 5 diphosphate (PIP2).

Further these studies have shown that the binding of PIP2 containing liposomes to aminoglycosides is in accordance with the established toxicity of aminoglycosides:

Neomycin > gentamicin > amikacin > neamine

ii) Liposomes have been used to understand the mode of action of local anesthetics. These studies have indicated that the action of a local anesthetic is mediated by the specific interaction with phosphatidylserine. These interactions probably cause significant membrane fluidization and displacement of Ca2+ from membranes. These two effects are also associated with anesthetic action of excitable membranes.

iii) Liposomes appear to be convenient in vitro models for studying the phototoxic effects mediated through active oxygen species, membrane damage and altered Ca fluxes.

5. In Cosmetics:

To investigate the use of liposomes in cosmetics, studies have been made to study interaction between skin and liposomes. According to the manufacturers, liposomes may deliver moisture and a novel supply of lipid molecules to skin tissue in a superior fashion to other formulations. In addition they can entrap a variety of active molecules and can therefore be utilized for skin creams, anti-aging creams, after shave, lipstick, sun screen and make-up.

6. In Textiles:

A new method of wool chlorination at pH < 7 using liposomes as vehicles for oxidizing agents is suitable for inhibiting or modulating the formation of cystic acid in wool fibers. The chlorination using liposomes inhibits the formation of cystic acid.

7. Other applications of liposomes:

The potential of making large quantities of inexpensive and stable liposomes may put forward several other applications. They range from water based paints, single tube two component glues or resins, self healing paints, and similar products. They are based mostly on the dissolving potential of liposomes and their ability to protect the encapsulated substance until an external stimulus such as the presence of oxygen, light, or change in hydration. Critical evaluation of these applications is difficult, however, because the information is mostly concentrated in progress reports, business analyses, or prospectuses of various producers. Photo cleavable lipid, such as naturally occurring plasmalogens, is cleaved by radicals generated in photosensitizer (zinc porphyrin,) under the adsorption of light quanta. In ecology, liposomes offer improvements in bioreclamation and various monitoring and analytical-diagnostic applications. For instance, it was shown that in an oil spill, the addition of various bacteria with possible nutrients encapsulated in liposomes improves the degradation rates of carbohydrates, which are otherwise very slow. Due to the surfactant action liposomes also improve the coagulation and sinking of oil spread on the water surface or its cleaning up with floating booms. The Environmental Protection Agency is testing liposomes' ability to deliver nutrients to oil spills to speed up the degradation. Liposomes containing membrane anchored chelators can be used to clean toxic or radioactive metals from solutions. For instance, water contaminated in a nuclear reactor can be purified by addition of such liposomes which could be easily precipitated after binding of the toxic ions. In addition to the above mentioned liposome applications there are many others which were not mentioned. In conclusion, it seems that liposomes established themselves as an important model system in several different basic sciences and as a viable alternative in several applications. The real future of liposomes is in anticancer and possibly other chemotherapies, gene therapy as well as some other medical applications such as artificial blood.

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