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A REVIEW ON LIPOSOMAL DRUG DELIVERY SYSTEM

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INTRODUCTION

The name liposome is derived from two Greek words: 'Lipos' meaning fat and 'Soma' meaning body. A liposome can be formed at a variety of sizes as uni-lamellar or multilamellar construction, and its name relates to its structural building blocks, phospholipids, and not to its size. Liposome are defined As "Liposome are simple microscopic vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecule." Various amphipathic molecules have been used to form liposome. The drug molecules can either be encapsulated in aqueous space or intercalated into the lipid bilayer.

Structural Components of Liposome

There are number of the structural and non-structural components of liposomes, major structural components of liposomes are.

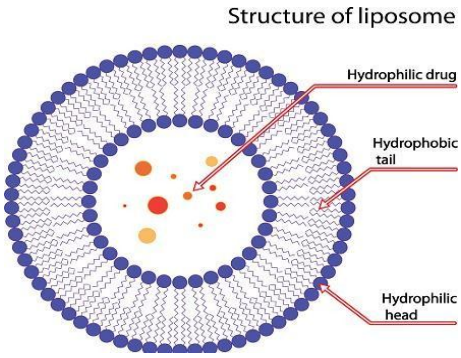
a. Phospholipids

Phospholipids are the major structural component of biological membranes, where two type of phospholipids exit- phospho diglycerides and sphingolipids. The most common phospholipid is phosphatidylcholine (PC) molecule. Molecule of phosphatidylcholine are not soluble in water and in aqueous media they align themselves closely in planner bilayer sheets in order to minimize the unfavourable 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 ration of cholesterol to phosphatidylcholine. Cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface and aliphatic chain aligned parallel to the acyl chains in the centre 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.



Advantages of liposomes

There are many drugs in the market, which have good therapeutics activities, but they are used in the dearest situation, because of their poor pharmacokinetics and pharmacodynamics activities. Drugs encapsulated in liposomes can be used regularly, as its pharmacokinetics and pharmacodynamics can be controlled. Some of the advantages of the liposome are as follows;

1. Provides selective passive targeting to tumour tissues (Liposomal doxorubicin).
2. Increased efficacy and therapeutic index.
3. Increased stability via encapsulation.
4. Reduction in toxicity of the encapsulated agents.
5. Site avoidance effect.
6. Improved pharmacokinetic effects (reduced elimination, increased circulation lifetimes).
7. Flexibility to couple with site-specific ligands to achieve active targeting

Disadvantages of liposomes

All drug delivery system has faults, same is the case of liposomes. As liposomes are required to enhance and increase the efficacy of drugs, the cost as well as all the other implications thereof must be taken into account. Cost is an issue when it comes to phospholipid preparation. This preparation is expensive to produce because of the costly raw material and equipment required for preparation. Liposomes are non-toxic but in the case of cationic liposomes, it tends to be toxic at higher concentrations.

Other problems related to liposomes are as following.

1. Sterilization: Sterilization of liposomes is a complicated process. Because it is unstable in heat and certain methods of radiation. Sterilizing with chemicals may affect stability problems. The only sterilization method is a membrane filter that is capable to filter liposomes of size <0.2µm.This method does not filter virus
2. Short self-life and stability: It is very difficult to achieve the stability of liposomal formulation due to chemical and physical degradation. Chemically, they are prone to oxidation and hydrolysis and they can physically fuse forming larger vesicles. It can be prevented by the addition of anti-oxidant such as tocopherol and the addition of cholesterol to avoid fusion.
3. Entrapment efficacy: The amount of drug a liposome can entrap is often low and sometimes leakage of drugs takes place.
4. Removal from circulation by the reticuloendothelial system (RES): The major drawback of liposomes as a drug carrier is that they are rapidly cleared by a phagocytic cell of the Mononuclear Phagocytic System (MPS). Larger liposomes are eliminated from circulation faster than smaller liposomes. PEGylation can increase the shelf life of liposomes.

TYPES OF LIPOSOMES

Liposomes are classified based on their structural properties, methods of preparation and composition, and application. Their properties such as the size of liposomes, number, the position of lamellae depend widely on the method of preparation, types of lipids used, and preparation condition of liposomes. This parameter, influence the in-vitro and in-vivo characteristics of liposomes.

The classification of liposomes based on structural properties is mentioned in Table-1, Classification based on liposomes preparation are mention in table-2,and based on composition and application are mentioned in table-3.

Table 1: Based on the structural parameter.	
MLV	Multilamellar vesicles,>0.5µm
OLV	Oligolammelar vesicles,0.1-1µm
UV	Unilammellar vesicles ,all size ranges
SUV	Small unilammellar vesicles,20-100µm
MUV	Medium sized unilammelar vesicles
LUV	Large unilammellar vesicles,>100µm
GUV	Giant unilamellar vesicles,>1µm
MV	Multivescular vesicles,>1µm

Table 2: Based on the method of preparation.	
REV	Single or oligolamellar vesicles are made by the reverse-phase evaporation method
MLV-REV	Multilamellar vesicles are made by a reverse-phase evaporation method
SPLV	Stable plurilamellar vesicles
FATMLV	Frozen and thawed MLV
VET	Vesicles prepared by the extrusion method
DRM	Dehydration-rehydration method

Table-3: Based on composition and application.	
Conventional liposomes (CL)	Neutral and negatively charged phospholipid and cholesterol
Fusogenic liposomes	Reconstituted sendai virus envelopes (RSVE)
PH-sensitive liposomes	Phospholipids such as PE and DOPE with earlier CHEMS or OA
Cationic liposomes	A cationic lipid with DOPE
Long-circulatory liposomes	Neutral high Tc□,cholesterol and 5-10%0f PEG- DSPE or GMI
Immuno-liposomes	CL or LCL with attached monoclonal antibodies or recognition sequences

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 hydrophilic head are attracted toward water where the head part faces toward the water and the hydrophobic tail are repelled by the water, when the lipid is exposed to aqueous environment, due to its amphipathic nature the phospholipid orient themselves to form bilayer where one layer of the phospholipid faces outside of the cells. Whereas another layer of the phospholipid faces inside the cell to avoid the water phase. The hydrocarbon tail of one layer faces the hydrocarbon tail of another layer and combines to form bilayer this structure is also called as lamella. Upon further hydration, the lipid cake (lamella) swells eventually that curves to form closed vesicles in the form of spheres known as liposome.

Method of Liposome Preparation and Drug Loading

Various method 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 or freeze drying
- Micro-emulsification
- Sonication
- French pressure cell
- Membrane extrusion
- Dried reconstituted vesicles
- Freeze-thawed liposomes

b. Solvent dispersion method

- Ether injection
- Ethanol injection
- Double emulsion vesicles
- Reverse phase evaporation vesicles
- Stable plurilamellar vesicles

c. Detergent removal method

- Detergent (cholate, alkylglycoside, Triton X100) removal form mixed micelles
- Dialysis
- Column chromatography
- Dilution
- Reconstituted sendai virus enveloped vesicles

2. Active loading technique

1. Passive loading techniques

a. Mechanical dispersion method

Lipid film hydration by Hand Shaking method

In this method, the lipid is solubilized in an organic solvent (mainly ethanol) in a round bottom flask with constant shaking in a circular manner, when the organic solvent evaporates, it forms a thin film of lipid on the RBF which on hydrated with purified water, with constant shaking, form a liposome. This method is useful for the preparation of MLV liposomes.

Nowadays, a Rotary evaporator machine is used for the formation of lipid film and hydration as it is more reliable than the handshaking method.

Non-shaking method

In these method lipid mixed with chloroform: methanol is spread over the conical flask and the solution is evaporated at room temperature without disturbing by the flow of nitrogen. After the solution gets dried it is hydrated by water-saturated nitrogen which is passed through the conical flask until the opacity of dried lipid film disappears. After hydration, the lipid gets swelled. Then the flask is inclined to one side and 10 to 20 ml of 0.2 M sucrose in distilled water is added to the side of the flask and then the flask is slowly returned to its original position. The fluid gently runs over the lipid layer on the bottom of the flask. Then the flask is flushed with nitrogen and sealed it was then allowed to stand for 2 h at room temperature. After swelling the suspension is centrifuged at 12000 g for 10 min at room temperature

Micro- emulsification

This method is also known as micro fluidization. Here microfluidizer is used to prepare small MLVs from concentrated lipid dispersion. The lipid can be introduced into fluidizers, either as a slurry of unhydrated lipids in organic medium or as a dispersion of large MLVs. Microfluidizer pumps the fluid at very high pressure through a 5 µm orifice and then it is forced along defined microchannels, which direct two streams of fluid to collide together at right angles at a very high velocity, thereby affecting an efficient transfer of energy. The fluid that is collected to be recycled through The pump in interaction chamber until vesicles of the spherical dimension is obtained after a single pass; the size of the vesicle is reduced to a size 0.1 and 0.2 µm in diameter.

Sonication Method: This is the most widely used method for the preparation of SUV from MLV, prepared from the handshaking method and rotary evaporator method. There are two types of sonication methods used in the preparation of SUVs.

a) Probe Sonication method

In this method, the tip of the titanium probe is directly dispersed into liposome dispersion for the production of SUVs. In this method, the energy input is high due to which there is the generation of heat. For controlling heat, liposome dispersion is kept in the ice bath. The main

disadvantage of this method is that the titanium fragment is sludge in a solution and contaminate it.

b) Bath sonication

In this method, liposome dispersion in a container is placed on the sonication bath. This method is more convenient as compared to probe sonication for the production of SUVs because the temperature can be controlled easily. The sterilized liposome can be obtained, there is no titanium contamination.

French Press method

In this method, unstable MLVs are converted to SUVs and LUVs bypassing then through a small orifice of equipment. Liposomes produced through this method are more reliable, as it has good stability as compared to those prepared by sonication method. The drawback of this method is that it has a small working volume of a maximum of 50 ml and a high temperature is hard to manage.

Membrane extraction

This method is carried out either with LUV or MLV. So initially, in these processes, the phospholipids are introduced in the buffered saline solution to form LUV or MLV. After that, the liposome is passed through polycarbonate membrane filter which results in uniform distribution of liposome which is of about 100 nm in diameter.

Freeze Thawed liposomes

Here, SUVs formed by the sonication method is frozen and thawed slowly and continuously, resulting in the formation of LUVs due to aggregation of SUVs during the thawing process. By this method, the encapsulation efficacies increase by 20-30%.

b. Solvent Dispersion method

Ether injection (solvent evaporation)

In this method, lipid dissolved in a diethyl-ether or ether-methanol mixture is gradually injected in an aqueous medium containing drug at the temperature of 50 to 65 ^{o}c or reduced pressure. The removal of ether under vacuum results formation of liposomes. The main drawback of this technique is the formation of a heterogeneous population of liposomes (70-200 nm) and exposure of liposomes in high temperatures during encapsulation which can hamper the stability of liposomes.

Ethanol injection

To a buffer a solution of lipid and ethanol is injected, resulting in the formation of MLVs. The drawback is the formation of a heterogeneous population of liposomes (30-110 nm). It is also difficult to remove ethanol from a solution consequently increasing the chances for the inactivation of biologically active macromolecules.

Double emulsion

In this method the active ingredient is mixed to the aqueous phase (w1) and then it was mixed with an organic phase to make a primary emulsion (w1/o) and then the primary emulsion is mixed with an aqueous phase to make a double emulsion w1/o/w2. The removal of the solvent leaves microspheres in the aqueous continuous phase, making it possible to collect them by centrifuging or filtering.

Reverse Phase evaporation method

This method has brought a breakthrough in the history of liposomes. The aqueous and lipid ratio used in this method is high, about four times higher than the handshaking method or MLVs. This method is based on the formation of reverse micelle where an aqueous medium is sonicated. The aqueous medium contains a water-soluble molecule to be encapsulated, lipids, and an organic phase. The slow elimination of organic solvent results in the formation of a gel-like consistency. At a critical point, the gel-like structure collapses to form liposomes.

c. 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.[34-36] 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).

In this method, removal of detergent in achieved by shaking mix micelle with beaded organic polystyrene absorbers such as XAD-2 beads (SERVA Electrophoresis GmbH, Heidelberg, Germany) and Bio-beads SM2 (Bio-Rad Laboratories, Inc., Hercules, USA).

Gel-permeation chromatography

In this method, the detergent is depleted by size special chromatography. Sephadex G-50, Sephadex G-l 00 (Sigma-Aldrich, MO, USA), Sepharose 2B-6B, and Sephacryl S200S1000 (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.

Dilution

Upon dilution of aqueous mixed micellar solution of detergent and phospholipids with buffer, the micellar size and the polydispersity increase fundamentally, and as the system is diluted beyond the mixed micellar phase boundary, a spontaneous transition from polydispersed micelles to vesicles occurs.

2. Active loading technique Industrial Production of Liposomes

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 areas 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.

(i) Detergent Dialysis

A pilot plant under the trade name of LIPOPREPR IICIS is available from Diachema, AG, Switzerland. The production capacity at higher lipid concentration (80 mg/ml) is 30 ml liposomes/minute. But when lipid concentration is 10-20 mg/ml 100 mg/ml then up to many litres of liposomes can be produced. In USA, LIPOPREPR is marketed by DianormGeraete.

(ii) Microlluidization

A method based on microemulsification /homogenization was developed for the preparation of liposomes. MICROFLUIDIZERR is available from Micro0udics Corporation, Massachusetts, USA. A plot plant based on this technology can produce about 20 gallon/minute of liposomes in 50-200 nm size range. The encapsulation efficiency up to 75% could be obtained. (iii) Aqueous dispersions of liposomes often have tendency to aggregate or fuse and may he susceptible to hydrolysis and or oxidation. Two solutions have been proposed.

(a) Proliposomes

In proliposomes, lipid and drug are coated onto a soluble carrier to form free-flowing granular material which on hydration forms an isotonic liposomal suspension. The proliposome approach may provide an opportunity for cost-effective large scale manufacture of liposomes containing particularly lipophilic drugs.

(b) Lyophilization

Freeze-drying (lyophilization) 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. The technique has a great potential as a method to solve long term stability problems with respect to liposomal stability. It is exposed that leakage of entrapped materials may take place during the process of freeze- drying and on reconstitution. Recently, 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 contents. It shows that trehalose is an

excellent cryoprotectant (freezeprotectant) for liposomes. Freeze-driers range in size from small laboratory models to large industrial units are available from Pharmaceutical Equipment Suppliers. Recently Schrier et al. (1994) have studied the in vitro performance of formulations prepared from lyophilized liposomes.

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.

Characterization of Liposomes

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

Physical characterization

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, grey colour indicates that presence of a non-liposomal dispersion and is most likely a disperse inverse hexagonal phase or dispersed micro crystallites. 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. Sepharose 4B and -2B columns can separate SUV from micelles.

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.

4. Liposome Stability

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.

5. 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.

6. Surface Charge

Liposome are usually prepared using charge imparting constituting lipids and hence it is imparting to study the charge on the vesicle surface. In general two method are used to assess the charge, namely free flow electrophoresis and zeta potential measurement. From the mobility of the liposomal dispersion in a suitable buffer, the surface charge on the vesicles.

Chemical characterization

It includes those assays which establish the purity and potency of various liposomal constituents. Liposomes is consist of fatty materials predominantly phospholipids. These phospholipids are composed of polar head (choline + phosphate), glycerol backbone and nonpolar tail (fatty acids). Hence the characterization of chemical nature of phospholipids plays a crucial role in quality of liposomes. Stewart discussed many techniques that involve the use of molybdate containing reagents yielding a blue-coloured product. Barlet assay (Detection at 830 nm), ascorbic acid assay (detection at 820 nm) are some of the examples in which molybdate is used. Phospholipids can also be analysed through complex formation with ammonium ferrothiocyante, (detection at 488 nm). Cholesterol concentration in liposomes can be analysed by assay of cholesterol assay method. Chromatographic methods are also used for chemical characterization. Formulations, especially in anticancer drugs, it is used by using HPLC. This method has an advantage of both separation and quantification of the constituents.

Biological characterization

Biological characterization is helpful in establishing the safety and suitability of formulation for therapeutic application. The biological characterization includes sterility testing, pyrogenicity testing and animal toxicity studies. Sterility testing of liposome is done to validate the presence/absence of viable microorganisms by using aerobic or anaerobic cultures. Pyrogen testing is usually done to determine bacterial toxins that may present in liposome. It is done by either by injecting drugs into rabbits or by limulus amoebocyte lysate (LAL) test. Animal toxicity is needed to characterize the safety and toxicity of liposome. It is done by using different parameters such as monitoring survival rates, histology and pathology of the animal tissues on which the prepared formulation is applied.

EVALUATION OF LIPOSOME

Evaluation is very important to ensure that the formulated liposome has the character required by the formulator. There are various evaluation test mentioned below.

1. Drug-excipient interaction study

This test is very important to ensure the compatibility of drugs and excipient in the formulation. This test is performed by the FTIR spectroscopy method. Here drug and excipient are mixed with KBr to form a sample pellet at the ratio of 100:1. In preparation of

pellets, a 5.5 metric ton of pressure is required to prepare a pellet. This pellet is scanned at the range of 4000-400 cm-1.

2. Drug loading study

Drug loading was determined spectrophotometrically. The drug quantification was confirmed by HPLC.

3. Lipid Quantification and chemical stability

Concentration and purity of phospholipids and cholesterol in the liposomes are determined by the HPLC method or enzymatically through cholesterol oxidase. TLC is also used to determine the hydrolysis of lipids at various stages of liposome preparation and storage.

4. Drug release determination

Drug release study of liposome is determined using the dialysis method. In a 250ml conical flask, 100ml of phosphate-buffered saline was taken.5mg lyophilized sample suspended in 1ml of PBS was taken into a dialysis bag. The bag is tight from both ends with threads and hanged inside a conical flask containing phosphate buffer which is stirred with the help of a magnetic stirrer under controlled temperature. The sample is taken out with the help of a micropipette, which is analysed at the spectrophotometer at the wavelength of 290 nm.

APPLICATION OF LIPOSOMES AS DRUG DELIVERY SYSTEM

To obtain a desired therapeutic efficacy and safety of drugs, a new drug delivery system has been formulated by a researcher. Liposomes are a new drug delivery system to achieve therapeutic efficacy and safety of drugs. Various application of liposomal formulation is mentioned below.

1. Site avoidance delivery

Many drugs are cytotoxic. On exposure of such drugs to a normal cell may cause an adverse effect, due to a low therapeutic index (TI). Such drugs when formulated to liposomes give therapeutic effect with low toxicity. For example, Doxorubicin causes cardiac toxicity, which subsides on the liposomal formulation.

2. Site-specific targeting

Site-specific targeting of drugs can be achieved by liposomal formulation. Here, drugs are encapsulated in a liposomal formulation, to which specific ligands are attached. This ligand

attached liposomes are very specific to a targeted cell. Which is very important for achieving a therapeutic effect at a desired site of action.

3. Intra-cellular drug delivery

Cytosol delivery of drugs can be achieved by liposomal formulation.N- (phosphonacetyl)L-aspartate (PALA) is normally poorly taken up into cells. Such drugs when encapsulated within liposomes, showed greater activity against ovarian tumour cell lines in comparison to free drugs.

4. Sustained release drug delivery

Drugs can be retained in a system for a prolonged time for the sustain release effect of drugs in a body. Drugs like cytosine Arabinoside can be encapsulated in liposomes for sustained release and optimized drug release rates in-vivo.

5. Reduced toxicity

Liposome formulation releases drugs for a prolonged period within the therapeutics index. The toxic effects of drugs can be reduced by50% in the case of liposome formulation. In anticancer drugs like doxorubicin, with cardiac toxicity, it can be reduced by the liposomal formulation of doxorubicin.

The various liposomal formulation for commercial use		
Drug	**Product**	**Indication**
Ambisome™	Amphotericin B	Fungal infection
DaounoXome™	Daunorubicin	Kaposi's sarcoma
Doxil™	Doxorubicin	Refractory Kaposi's sarcoma, recurrent breast cancer, and ovarian cancer
Visudyne®	Verteporfin	Age-related macular degeneration, pathologic myopia, and ocular histoplasmosis
DepoCyt®	cytarabine	Neoplastic meningitis and lymphomatous meningitis
Myocet®	doxorubicin	Recurrent breast cancer
Lipoplatin®	cisplatin	Epithelial malignancies

The liposomal formulation of doxorubicin

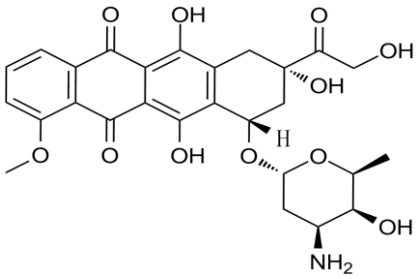
Doxorubicin is the best known and most widely used member of the anthracycline antibiotic group of anticancer agents. It was first introduced in the 1970s, and since that time has become one of the most commonly used drugs for the treatment of both haematological and solid tumours. The therapy-limiting toxicity for this drug is cardiomyopathy, which may lead to congestive heart failure and death. Approximately 2% of patients who have received a

cumulative (lifetime) doxorubicin dose of 450–500 mg=m2 will experience this condition. An approach to ameliorating doxorubicin related toxicity is to use drug carriers, which engender a change in the pharmacological distribution of the drug, resulting in reduced drug levels in the heart. Examples of these carrier systems include lipid-based (liposome) formulations that effect a beneficial change in doxorubicin biodistribution, with two formulation.

Doxorubicin

Chemistry

Doxorubicin is an anthracycline antibiotic originally isolated from Streptomyces peucetius var. caesius.



The molecular formula of the drug is C27 H29 NO11·HCl; its molecular weight is 579.99. This amphipathic molecule possesses a water-insoluble aglycone (adriamycinone: C21H18 O9) and a water-soluble, basic, reducing amino-sugar moiety (daunosamine: C6H13 NO3)s approved for clinical use.

Doxorubicin hydrochloride typically exists as a hygroscopic crystalline powder composed of orange-red thin needles. It has a melting point of 229–231 and absorption maximums (in methanol) of 233, 252, 288, 479, 496, and 529 nm due to the dihydroxyanthraquinone chromophore.

Mechanism of Action

Doxorubicin interacts with DNA by intercalation and inhibition of macromolecular biosynthesis. This inhibits the progression of the enzyme topoisomerase II, which relaxes supercoils in DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication.

Pharmacokinetics

Absorption- Absorb rapidly after intravenous administration.

Distribution-Widely distributed in the body. Large amounts are seen in liver, kidney and heart.70% bound to the plasma proteins.

Metabolism- Metabolized in the liver to its metabolites. Doxorubicinol is its major metabolite.

Excretion- Excreted via bile. A small portion is excreted through urine.

Pegylated liposomal doxorubicin (Caelyx/Doxil) & liposomal daunorubicin (DaunoXome) produce lower peak plasma concentrations and longer circulation times than free drug Liposomal doxorubicin in Myocet has systemic availability, metabolism, and excretion similar to that of conventional doxorubicin, but at a slower rate.

Stealth liposomes

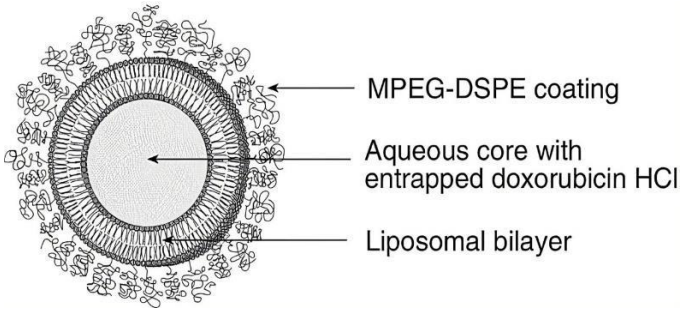
Doxorubicin HCl encapsulated in long-circulating STEALTH® liposomes. Liposomes are microscopic vesicles composed of a phospholipid bilayer that are capable of encapsulating active drugs. The STEALTH® liposomes are formulated with surface-bound methoxy polyethylene glycol (MPEG), a process often referred to as pegylation, to protect liposomes from detection by the mononuclear phagocyte system (MPS) and to increase blood circulation time.

These stealth liposomes are spherical vesicles with a membrane composed of phospholipids bilayer used to deliver drugs or genetic material into the systemic circulation. Moreover, these types of liposomes were composed of various polymers like Polyethylene glycol (PEG), Polyaniline (PA), polyacrylamide (PAA), Poly vinyl pyrrolidine (PVPA) etc. These polymers were used to enclosed or to form the outer membrane of the liposomes.

Fate of stealth liposomes

Conventional liposomes are taken up by the reticular endoplasmic system (RES) and they are liable for degradation or inactivation by the phagocytosis. Conventional liposomes entering in the blood stream unable to target the tumour site as they lack essential binding mechanism, by which they linked to tumour receptors and attack. This is the area of stealth liposomes show the concept of formulation. Stealth liposomes, due to presence of PEG derivatives on outer membranes provide stealth effect i.e. they are not detected by the phagocytes system. Eventually they will detect and eaten. But this process is too slow. And hence they provide long circulation time. Inability of detecting these liposomes by reticular endoplasmic system

they resemble the stealth bombers, so they named after them. These stealth liposomes provide accurate, precise attack on the cancer cells and deliver the drug molecule at the site of action.



Representation of Stealth liposome

Enhanced permeability and retention activity of stealth liposomes

Conventional liposomes on administration reach to the site of action through systemic circulation. Due to smaller size in µm they passively diffuse through the leaky walls of the blood vessels and reaches to the tumour site. Conventional liposome has permeability through cancer cells but has no such retention effect as there is no binding mechanism on outer surface. Due to this inability of binding to tumour site, conventional liposomes are proven inefficient in treating the tumour cells. On the other hand, Stealth liposome has smaller size up to ~50um and has PEGylated molecules attached to the outer side of the stealth liposome, providing better binding site to the cancer cell. PEGylation of stealth liposome mimic the host cell properties. And with the PEG it is to pass the leaky blood walls and reaches to cancer cell. This improves the retention activity of the drug molecules. Stealth liposomes provide targeted drug delivery and also provide bulk drug transfer at the tumour site.

Characteristic of stealth liposome

1. Stealth liposome is composed of cholesterol and phospholipids such as phosphotidyl choline or diacetyl phosphate, the composition and structure remains the same as in the host cell.
2. The phospholipids bilayer consists of hydrophilic head component and hydrophobic tail and PEG or other polymer as outer coat.
3. Stealth liposomes are stable in nature.
4. The size, shape of stealth liposome can be altered depending on drug and material used.
5. They can’t be taken by endoplasmic reticular system and cause slow release of drug.
6. There size ranges from 50 to 5000nm.

7. The lipids most commonly used are phospholipids, sphingolipids, glycolipids and sterols.
8. The stealth liposomes are colloidal and uniform in nature.

Advantages of Stealth liposome

- Increased bioavailability
- Stealth liposome provides long, slow release.
- Toxicity and side effects are minimized.
- PEGylated liposomes can offer some targeted delivery
- Passive targeting occurs for tumours and inflamed tissues.

Preparation of liposomes

Materials required

For preparation of liposomes

1. Di stearoyl phosphatidylcholine (DSPC), cholesterol (CHOL), distearoyl phosphatidylethanolamine-N-monomethoxy polyethylene glycol (Mw 2,000) (mPEGDSPE) Lipids. All lipids were stored at under –20◦C. Prior to weighing, lipids should be brought to room temperature.
2. Doxorubicin is weighed .As with the handling of any cytotoxic agent, gloves and masks must be used when weighing doxorubicin.
3. Hydration buffer: pH 4.0, 0.3 mol/L citrate buffer, 39.35 g citric acid (Mw 210.1), and 33.15 g sodium citrate (Mw 294.1) dissolved in deionized water to make 1,000 mL. Store at room temperature.
4. Base for adjusting liposomes external pH: 0.5 mol/L sodium carbonate, 52.995 g sodium carbonate (Mw 105.99) dissolved in deionized water to make 1,000 mL. Store at room temperature.
5. 0.9% Sodium chloride (NaCl).
6. High-pressure homogenizers.

For Phosphorus Assay

1. Dipotassium hydrogen phosphate trihydrate (Mw 228.24) standard solution: 18.418 mg dissolved in deionized water to make 25 mL. At this concentration, 10 µL standard solution contains 1 µg phosphorus. Store at room temperature.
2. Perchloric acid (70% w/w), hazardous, handle with caution, wearing gloves, in fume hood.

3. 2.5% Ammonium molybdate: 2.5 g ammonium molybdate dissolved in 100 mL diH2O, freshly made
4. 10% Ascorbic acid: 10 g ascorbic acid dissolved in 100 mL diH2O, freshly made, prevent from light.
5. UV–VIS Spectrophotometer, UV-2401.

For Transmission Electron Microscopy.

1.2% Phosphotungstic acid (PTA):2 g phosphotungstic acid dissolved in 100 mL diH2O, adjust pH to 7.0 with KOH. Store at 2–8◦C.

2. Hitachi H-7600 Transmission Electron Microscope.

For Size Determination

Submicron Particle Sizer

For Encapsulation Efficiency

1. Doxorubicin liposomes lysis solution: 90% isopropanol containing 10% 0.075 mol/L hydrochloride.
2. Sephadex G50 (medium), 4 g power added into 200 mL diH2O or the external buffer used for the liposomes, boiled for 1 h, cooled down to room temperature.
3. Column, 1.5 cm × 15 cm, clean and intact with the nets, net fasteners, silicon tubes.

In Vitro Release

1. Drug release buffer: pH 7.4, 10 mM PBS, 150 mM NaCl, and/or containing 1% human serum.
2. Dialysis bag, cut off size 8,000–12,000, immersed in boiled diH2O for 30 min, rinse with PBS buffer. 3. F-3000 spectrofluorometer.

METHODS

Preparation of Doxorubicin Liposomes

1. Weigh 160 mg DSPC, 39.2 mg cholesterol, 39.8 mg MPEG2000-DSPE (with mole ratio 2:1:0.14) using an analytical balance, dissolve in 10 mL chloroform in a 50-mL roundbottom flask.
2. The round-bottom flask was set in a rotary evaporator, the organic solvent was evaporated under reduced pressure, and the flask was seated in a 30–35◦C water bath. And after a dried

thin film formed on the wall of the flask, the flask was further dried under vacuum for 4–6 h to eliminate the residual solvent.

3. The dry film was hydrated with 10 mL, pH 4.0, 0.3 mol/L citric buffer, vortex 5 min, and then shaking 1 h at 65◦C. Multi-layer vesicles (MLV) were formed.

4. The size of the formed MLV was reduced by five freeze– thaw cycles using liquid nitrogen and 65◦C water bath. Wear eye protection goggles and cotton gloves.

5. Clean the homogenizer with phosphate-free soap water, hot tap water, ethanol, and distilled water and last the hydration buffer. Maintain the extruder at 65◦C (Note 1).

6. The MLV, after the freeze–thaw cycles, was extruded through two stacked 0.4 μm and then through 0.1 μm pore-diameter polycarbonate filters using high-pressure extruder device (3, 4), each extrusion step was performed 8–11 times at 65◦C. And large unilamellar vesicles (LUV) were formed. 7. The prepared liposomes (volume V0 with lipids concentration C0) pH was adjusted with that of the outer liposome pH value to pH 6.5–pH 7.0 with 0.5 mol/L sodium carbonate. Write down the volume of sodium carbonate used (V1) and the lipid concentration was estimated by the formula: C1 = V0 × C0/(V0+V1). The precise lipid concentration was determined by phosphorus assay, which was described in Section 3.2.1 (Note 2).

8. Preheat proper volume DOX (10.0 mg/mL in 0.9% sodium chloride, Drug/Lipid = 1:5– 10 mole ratio) to 65◦C, mix with 5-mL liposome solution with outside pH 6.5–7.0, shaking 20 min at 65◦C (Note 3).

9. DOX liposome was prepared.

Liposome Characterization Analysis

This section includes the following several parts.

- Phospholipids concentration determination by phosphorus assay
- Liposome morphology by transmission electron microscopy
- Liposome-size distribution
- Liposomal encapsulation efficiency
- In vitro release

Phospholipids Concentration Determination by Phosphorus Assay

1. Take 0, 10, 20, 30, 40, and 50 μL dipotassium hydrogen phosphate trihydrate standard solution into a 15-mL glass tube with glass stopper, triplicate, and add deionized water to each tube to make 50 μL in total (5).

2. Add 0.5 mL perchloric acid (70% w/w), mix, and heat in oil bath for 4.5 h at 130◦C, manipulate in hood.
3. The tubes were cooled down to room temperature, and added the fresh cocktail containing 3.0 mL deionized water, 1 mL 2.5% ammonium molybdate, and 0.5 mL 10% ascorbic acid. Mix well immediately.
4. Incubate 1.5 h at 37◦C, shaking. Then cool down to room temperature.
5. Reading the absorbance at 822 nm wavelength via UV–VIS spectrophotometer. A standard curve of absorbance versus phosphorus concentration is obtained.
6. Take 10–50 µL liposome sample and add deionized water to make 50 µL, and then follow the above Steps 2–5.
7. The lipid concentration was calculated according to the standard curve

Liposome Morphology by Transmission Electron Microscopy (TEM)

1. Place one drop of liposome specimen onto a sheet of parafilm.
2. Place plastic/carbon-coated 400-mesh copper grid (film face down) on drop and let absorb for approximately 10 min.
3. Wick away excess fluid with filter paper.
4. Stain: Place grid (plastic-side down) on drop of filtered 2% PTA, pH 7.0, and let stain for 1–2 min.
5. Wick away excess fluid with filter paper, and place grids, specimen-side up, in specimen petri dish, and further dried in air for 30 min.
6. The specimens were observed using a Hitachi H-7600 Transmission Electron Microscope operating at 80 kV.

Size-Distribution Analysis

1. DOX liposome samples of 0.2 mL was diluted to 1.0 mL with 0.9% NaCl (filtered through 0.2 µm membrane), generally, phospholipid concentration was in the range of 2–10 mg/mL and analysed by Submicron Particle Sizer (6).
2. Turn on the Submicron Particle Sizer (Nicomp 380 ZLS, Particle Sizing Systems, Inc., Santa Barbara, CA) and turn on the computer and then the ZW 380 software.
3. Click “setup” on the menu, choose “Fixed angel 90 Deg,” click OK, and “Correlator is connected” shows up.
4. Click “Particle sizing” on the menu, and choose “Control menu,” “Autoprint/save menu” to set the measuring parameters and the file path.

5. Transfer the diluted sample into the small glass tube by pipette, the intensity reading of the sample should be around 300.
6. Choose "Vesicle" style for liposome-size measuring from the icon tools.
7. Print the size and its distribution. Generally, if Chi-squared is under 3, Gaussian distribution is used, and when Chisquared is larger than 3, Nicomp distribution is used. And the volume-weighting is often used for liposomes.

Liposomal Encapsulation Efficiency

1. Swell 4 g of Sephadex G50 medium with 200 mL deionized water in a boiled water bath for 1 h. Prepare a media slurry in a ratio of 70% settled gel to 30% buffer. Equilibrate to room temperature.
2. Prepare the column 1.5 cm × 15 cm, make sure that the nets, net fasteners, and glass tube are clean and intact. Mount the column vertically on a laboratory stand. Fill the column with a few millilitres of buffer using the syringe from the bottom, pour the media slurry into the column, down a glass rod against the wall of the column, in one continuous motion, and equilibrate the column with 100 mL buffer.
3. Add 1 mL liposome sample on the top of the column, let it run into the gel, and then add a few mL buffer to the column, elute with buffer, collect DOX liposomes, write down the volume (Section4)
4. Dissolve 0.1 mL DOX liposome sample before and after the column with 0.9 mL of 90% isopropanol containing 10% 0.075 mol/L HCl, measure absorbance at 480 nm by UV–VIS spectrophotometer.
5. Liposomal DOX encapsulation efficiency (EE%) is EE% = [A1 × V1/A0] × 100%

A1: The absorbance of the sample after the column

A0: The absorbance of the sample before the column

V1: The volume obtained from 1 mL sample running the columnar liposomes.

In Vitro Drug Release

1. Prepare the standard curve of fluorescence versus DOX concentration in PBS. The DOX standard solution of 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 μg/mL in PBS, triplicate, fluorescence was measured at excitation wavelength λEx = 501 nm and emission wavelength λEm = 555 nm. The standard curves of DOX in 1% human serum–PBS and 0.075 mol/L HCl–90% isopropanol were the same as in PBS, except DOX dissolving in the corresponding buffer (8).

2. Cut dialysis bag (cut off size 8,000–12,000) with 5 cm length, hydrated with PBS buffer. Seal at one end, add 1 mL DOX liposomes into the bag, and seal the other end.
3. Put the bag into a 50-mL round-bottom flask, filled with 50 mL PBS, triplicate each sample. Shake the flasks at 37◦C at 100 rpm.
4. At 0, 0.5, 1, 2, 3, 5, 7, and 10 h, take 1 mL sample from the flask, and add 1 mL PBS at the same temperature to the flask. Measure fluorescence at excitation wavelength λEx = 501 nm and emission wavelength λEm = 555 nm.
5. At the end point, cut the dialysis bag and release all samples to the flask, as total released concentration after calibration with DOX content taken out of each point.
6. Drug release curve can be obtained by release percentage versus time.

CONCLUSION

There are many drugs molecule which is having good pharmacological action but their use are limited due to the toxicity, they posses. Such drugs can be brought into use by reducing its toxicity and enhancing its pharmacological action. Liposome formulation is an appropriate approach to achieve the therapeutic action of such drugs. Liposome composition has made it more reliable as it is inert and resembles a cellular membrane which makes it an interesting field of research for scientists. A liposome is a good carrier of drugs in the treatment of cancer and it is gaining popularity in the field of chemotherapy. An anticancer drug, doxorubicin is prepared as stealth liposomes. Stealth liposomes are the new drug delivery systems which were recently developed to overcome the errors encountered in the conventional liposomes. The systems, due to their advantage of fooling the phagocytic system i.e. MPS, provide the accurate and efficient drug delivery at the site of action. These types of targeted drug delivery systems were now developed to improve the medication and pharmaceutical product to improve the dosage form.

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