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Mini-Review On Liposomes: Composition, Methods Of Preparation, Studies Explaining Significance Of Extrusion, Freeze Drying And Biological Evaluation Of Liposomes.

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Abstract:
Liposomes are amongst the various nano-carriers that are used to target drugs to a specific tissue. They are colloidal, vesicular structures of phospholipid molecules that are surrounded by equal number of aqueous compartments. The lipid bilayer encapsulates an aqueous interior which can be loaded with cargo such as peptides and proteins, hormones, enzymes, antibiotics, antifungal and anticancer agents. This structural property of liposomes makes it an important nano-carrier for drug delivery. Extrusion is currently one of the most common methods for producing monodisperse unilamellar liposomes with uniform sizes. Typically, a lipid suspension is forced through a polycarbonate membrane with a well-defined pore size to produce vesicles with a diameter that's close to the pore size of the membrane. The unilamellar vesicles can thereby be loaded with therapeutic moieties, freeze dried and evaluated for biological activity summing up the goal of this mini-review article.

Keywords: *Liposomes, Phospholipids, Drug delivery, Extrusion, Freeze Drying*

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I. Introduction:

The process of drug design widely recognizes the critical role of drug delivery systems and thus a great number of controlled drug delivery systems have been developed in recent years. Their main function is to deliver drugs at the desired site in a desired rate to maximize the drug efficacy and minimize side effects.[2,24] Liposomes are small artificial spherical vesicles whose particle size ranges from 30 nm to several micrometers.[3] Structurally they consist of a lipid bilayer which are surrounded by aqueous units. They further consist of a polar head group which tends to be oriented in the pathway of interior and exterior aqueous phases. Lipids are amphipathic in aqueous media hence they can be used to transport both hydrophilic and hydrophobic drugs.

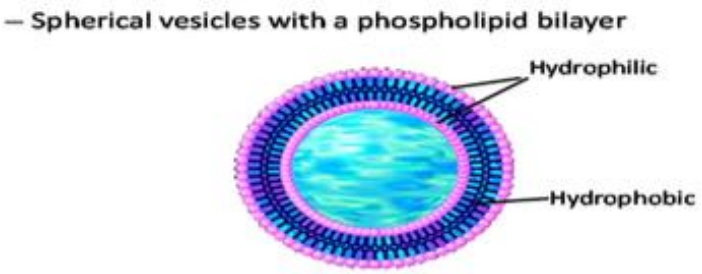

Figure 1: General structure of Liposomes[3]

A brief review of the advantages and disadvantages of liposomes reported in literature:

Advantages of Liposomes[3,4]:

- Liposomes are biocompatible and completely biodegradable. They are also non-toxic and non-immunogenic.
- Due to their amphipathic nature, they can deliver both hydrophobic and hydrophilic drugs
- Drug encapsulation in liposomes is possible which thereby enables protection from external environment.
- Liposomes help reduce the exposure of sensitive tissues to toxic drugs.
- Flexibility to couple with site-specific ligands to achieve active targeting.

- Liposomes increases efficacy and therapeutic index of drugs (Actinomycin-D)

Disadvantages of Liposomes[3,4]:
- Liposomes have low solubility.
- They possess a short half-life.
- Leakage and fusion of encapsulated drug/molecules.
- Their production cost is high.

There are several studies conducted on liposomes with the goal of decreasing drug toxicity and/or targeting specific cells. A review by Akbarzadeh et al.[3] mentions the use of Liposomal encapsulation technology which is the latest delivery technique used to transmit drugs that act as promoters to targeted organs. This delivery techniques helps to form a barrier around the encapsulated drug and thus helps in protecting the integrity of the formulation against enzymes, digestive juices, bile salts and intestinal flora. Feng et al.[2] worked on the fabrication and characterization of liposomes in microsphere of biodegradable polymer. Few conclusions of this study included a liposome encapsulation efficiency of 50% in polymeric microspheres. Additionally, they also achieved invitro release control by adjusting the composition of liposomes, the polymeric materials, and the microencapsulation process parameters. Liposomes certainly have a variety of applications in drug therapy especially in cancer therapy. Cancer is the uncontrolled growth of cells, which occurs due to the accumulation of genetic mutation and aberrant signaling of the cells[5]. Chemotherapy is used as a treatment option but has limitations with respect to drug bioavailability and pharmacokinetics. To overcome these limitations of conventional chemotherapy, several nanocarrier delivery systems are being developed. Liposomes are one of the nanocarriers used to target drugs to the tumor site[24].

Classification and Preparation of Liposomes:
Liposomes are classified majorly based on size and the number of phospholipid membrane layers[5,25].

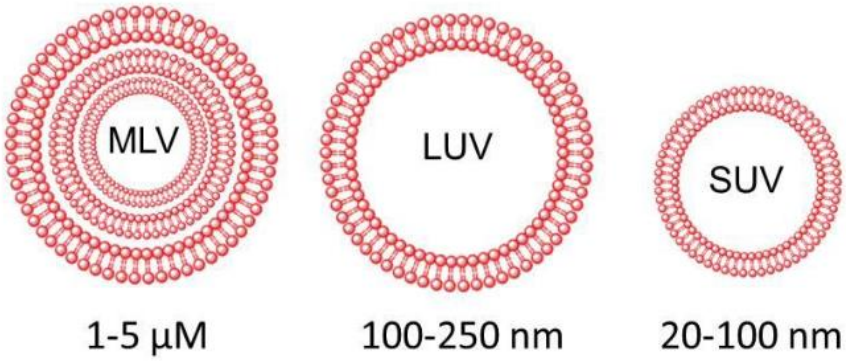

Figure 2: Classification of Liposomes[5]

Multilamellar Vesicles (MLV): They consist of number of concentric phospholipid bilayer membrane separated by aqueous phase. Structurally they are big in size and the observed sizes are close to 5µm[25].

Small Unilamellar Vesicles (SLV): These liposomes are composed of aqueous compartments enclosed by a single lipid bilayer. Size of these liposomes are in the range of 20-100 nm.

Large Unilamellar Vesicles (LUV): They are also composed of a single lipid bilayer surrounding aqueous compartment but have a larger size in the range of 100-250 nm.

A review by Sharma et al.,[6] describes how liposomes can be classified based on composition. They are composed of natural and/or synthetic lipids (phospho- and sphingo-lipids) and may also contain other bilayer constituents such as cholesterol and hydrophilic polymer conjugated lipids. Liposomes can be classified in terms of composition and mechanism of intracellular delivery into 5 types as i) Conventional Liposomes (CL) ii) pH-sensitive liposome iii) cationic liposomes iv) immune liposomes v) long-circulation liposomes.[6]

General methods of liposome preparation include a) Drying lipids from organic solvent b) Dispersing lipid in aqueous medium c) Purifying the resultant liposome d) Analyzing the final product. Liposomes are generally prepared using various procedures in which the water soluble (hydrophilic) materials are entrapped by using aqueous solution of these materials as hydrating fluid or by the addition of drug/drug solution at some stage

during manufacture of the liposomes.[7] Preparation of liposomes depends on the following parameters: a) Physicochemical characteristics of the materials to be entrapped and those of the liposomal ingredients.

b) the nature of the medium in which the lipids are dispersed c) effective concentration of the encapsulated substance and its potential toxicity d) optimum size, polydispersity, and shelf life of the vesicles for the intended application e) production reproducibility and large-scale production of safe and efficient liposomal products. Fabrication of liposomes is based on Active and Passive loading techniques.[7] Passive loading includes Mechanical dispersion, Solvent dispersion, and Detergent removal methods. Active loading is based on the encapsulation property of drugs.

Mechanical dispersion method:

Sonication: It is the most extensively used method for preparation of SUV. Additionally, MLV's are sonicated with a bath type of probe sonicator. Cup horn sonicators which are less widely used have successfully produced SUV. Bath sonicators are most widely used for preparation of SUV.[7] Disadvantages of sonication include low 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.

French pressure cell method: This method involves the extrusion of MLV through a small orifice. The method is simple, rapid, reproducible and involves gentle handling of unstable material making it more advantageous than sonication. Some disadvantages of this technique are that the high temperature is difficult to attain high temperatures and the working volumes are comparatively small.[3]

Freeze-thawed liposomes: A study by Traika et al.[8] describes the formation of MLV, SUV and LUV by freeze thawing. MLV's were prepared by mixing phospholipids with chloroform. SUV were prepared by sonication of MLV dispersion using probe type sonicator. LUV's were prepared by reverse phase evaporation technique.

Solvent dispersion method:

Ether injection: This method involves the dissolution of lipids into ether or ether-alcohol mixture thereby gradually injecting it into aqueous solution of materials to be encapsulated. The temperature range usually maintained is 55-65°C. Major disadvantages of this technique is that the population being heterogeneous (70-190 nm) and the compounds to be encapsulated are exposed to organic solvents at high temperature.[7]

Ethanol injection: MLV's are formed when a lipid solution of ethanol is rapidly injected to a huge excess of buffer. Disadvantages of this method includes the population being heterogeneous (30-110 nm), liposomes are very dilute, the removal of ethanol is difficult because it forms azeotrope with water and inactivation of biologically active macromolecules occurs in the presence of ethanol.[3]

Reverse phase evaporation method: This method allows the preparation of liposomes with a high aqueous space to lipid ratio and a capability to entrap a percentage of aqueous material presented.[3] It utilizes solvents such as diethyl ether/ isopropyl ether or mixture of diethyl ether and chloroform (1:1 v/v) and a mixture of chloroform-methanol (2:1 v/v) containing phospholipids. The organic phase is immiscible with aqueous phase leading to formation of o/w type emulsion. To improve the efficiency of liposome formulation Phosphate-buffer saline or Citric-Na_2HPO_4 buffer is added to the aqueous phase. Formation of liposomes take place by rotary evaporation of organic solvents.[9] The main advantage of this method is high encapsulation rate, and the drawback is residual solvent in the formulation.[9]

Supercritical fluids in the Preparation of Liposomes: Supercritical fluids are used to overcome the existing problems posed by conventional methods such as use of highly toxic solvents and limited laboratory preparation.[10] Supercritical carbon dioxide is the most used fluid in the preparation of liposomes. It has several advantages as non-toxicity, non-flammability, recyclable and easy removal from solvent, operation at moderate temperature and avoiding degradation at inert atmosphere.[10] This method allows controlling the extraction condition by variation of temperature, pressure or adding modifier solvents as co-solvents.

Extrusion Technique: Extrusion is currently one of the most common methods for producing monodisperse unilamellar liposomes and controlling their sizes.[11,26,27] Typically a lipid suspension is forced through a polycarbonate membrane with a well-defined pore size to produce vesicles with a characteristic diameter near the pore size of the membrane used in preparing them. An advantage of this technique is that there is no need to remove the organic solvent or detergent from the final preparation. A study by Lapinsky et al.,[11] explains the procedure of liposome preparation by extrusion which involves chloroform being evaporated from lipid solution subsequently drying and hydrating the film using buffer containing NaCl. Their goal was to obtain a final lipid

concentration of 1mg/ml. The mixture was processed 5 times through a freeze-thaw-vortex cycle to ensure complete mixing. Each cycle consisted of immersing in liquid nitrogen for 5 minutes and a second immersion in 60°C water bath for 5 minutes followed by vortexing the thawed sample for 2 minutes. Next step included extruding through 400nm pore diameter polycarbonate membrane filters using a miniextruder. The sample extrusion was done 11 times through 2 polycarbonate membranes with nominal pore diameter of 100nm at room temperature.[11]

Studies involving Extrusion of Liposomes:

Study 1:

A study by N. Berger et al.,[12] describes the filter extrusion of liposomes using different devices by comparing liposome size, encapsulation efficiency and process characteristics. Preparation of liposomes was by extrusion and subsequently passing them through 5, 1, 0.4, 0.2, 0.1 and 0.05 μm pore sizes using two different filter-extruders, the continuous high pressure Dispex Maximator® (CE) or alternatively the discontinuous Avestin Liposofast® (DE). For this study, comparison of particle size, lamellarity and encapsulation efficiency of Calcein dispersions were conducted. Due to high flow rate and pressure drops the liposomes produced by CE were smaller than DE except for final filter pore size (0.05 μm). For bigger liposomes, the Nicomp 370 revealed bigger volume based mean particle sizes compared to Malvern Zetasizer. Small liposomes extruded through 0.05 μm filters yielded similar liposome sizes irrespective of whichever microscopic technique being used. Compared to size of liposomes, encapsulation efficiencies were smaller for CE than DE at all filter stages except the final (0.05 μm). Also, there was no lipid loss observed for all types of extrusion techniques being used.[3]

Study 2:

Another study by Castile and Taylor [13], describes various factors affecting the size distribution of liposomes produced by freeze-thaw extrusion. The study was aimed to develop a protocol to produce liposomes using a freeze-thaw extrusion methodology. The particle size was analyzed by laser diffraction particle size analyzer. Particle size was increased when cholesterol was included in the bilayer of MLV's. Use of Poloxamer P338 and P407 inhibited the increase in size. A key observation in this study was that dispersing the liposomes in sodium chloride solution promoted size increase following freeze-thawing suggesting that vesicles had aggregated or fused.[4]

Study 3:

A study by J.O. Eloy et al.,[14] describes the development, characterization and in-vitro and in-vivo evaluation of Paclitaxel/Rapamycin co-loaded liposomes for breast cancer therapy. Both Paclitaxel and Rapamycin provide synergistic effect against breast cancer. The liposomes were prepared by the hydration of thin lipid film. The composition based on SPC:Chol:DSPE-PEG (2000) was employed for both Paclitaxel and Rapamycin-loaded liposomes. Briefly, lipids and drugs were dissolved in chloroform, which was evaporated in rotary evaporator for 30 min at 65°C. Then, the lipid film was hydrated with phosphate buffer (pH 7.4) during 30 min at 100 rpm. The formulations were extruded 5 times in a 0.2 μm membrane and 3 times in 0.1 μm membranes, filtered (0.45 μm) in Polyvinylidene difluoride membranes and purified by gel filtration chromatography in a sepharose CL-4B column. Particle size was determined by Dynamic light scattering and zeta potential was determined by Zetasizer 3000 HSa. FTIR was used to assess the drug loading content. TEM was used to evaluate the shape of liposomes. Some key conclusions of this study were that both drugs were active and soluble in their amorphous form. The formulation showed excellent colloidal stability and retained drugs encapsulated with sustained release. The in-vitro 4T1 breast cancer cell lines and in-vivo 4T1 tumor-bearing mice showed that the drugs acted synergistically with promising tumor growth control.[14]

Study 4:

A study by N.P. Aditya et al.,[15] describes the preparation characterization and antimalarial activity of Curcuminoids-loaded liposomes in combination with artether against Plasmodium berghei infection in mice.

The liposomes were prepared by the modified thin-film hydration method. A range of molar ratios of curcuminoids to lipid (1:5, 1:7.5, 1:10) were tested. Soy PC and hydrophobic drug curcuminoid were dissolved in 2:1 chloroform: methanol mixture. The solvent was evaporated by rotary evaporator. The lipid film was then hydrated at 45°C using phosphate buffer saline (pH 7.4) under continuous shaking at about 200g for 10 min. The homogenous liposomal dispersion was downsized by passing through 100 nm polycarbonate membrane (11times each) using an extruder to obtain uniformly sized liposomes which were subjected to physicochemical characterization. Size distribution profile of liposomes was determined by using photon correlation spectroscopy. Curcuminoids were quantified by using HPLC method. The physicochemical characteristics of prepared liposomes were as per expectations. Since a major drawback of curcumin loaded liposomes was its inability to provide a complete cure, combination therapy was employed. Combination therapy gave complete protection

upto 50 days without recrudescence in mice. This strategy has certain advantages, since arteether is used clinically for malaria, it also has high clearance rate and needs to be combined with long-acting anti-malarial drug.

Freeze-drying of Liposomes (Lyophilization):

Lyophilization also known as freeze-drying is an efficient way to stabilize liposomes which thereby increases the shelf life of liposomes especially those containing thermosensitive drugs.[16,28] This method involves the removal of water from products in the frozen state at tremendously low pressures. Lyoprotective mechanisms such as water replacement and vitrification models have been known for the past few decades and are well recognized. The protective effect during liposome lyophilization is mainly determined by the formulation factors such as the nature of the drug, the lipid bilayer composition and the choice of lyoprotectants. Optimization of these factors is an efficient way of improving the stability of these lyophilized liposomes. The solubility and partitioning characteristics of drugs determines its permeability to enter the lipid bilayer. Highly lipophilic drugs can stay in the bilayer comfortably compared to the hydrophilic/amphiphilic drugs.[16] Additionally, Chen et al.,[16] reviews the lyophilization of surface-modified liposomes which is being widely investigated due to their useful properties eg. high therapeutic efficiency, low side effects, longevity in the body, targeting the site of disease, contrast properties for diagnostics. The effect of freezing protocols on the stability during lyophilization is mainly determined by factors such as the freezing rate, freezing temperature and ice nucleation rate. Generally, four different freezing protocols are used for lyophilization:[16,17] a) Freezing in liquid nitrogen at 250°C/min b) Freezing down to a low temperature (-45°C or other) at 0.6-1°C/min c) Freezing in liquid nitrogen and annealing at low temperature (-20°C) for few hours. d) Freezing with a precooled plate down to a low temperature (-45° C) at 1-2°C/min.

The selection, amount and combination of lyoprotectants such as glycols, vitamin E and phosphates have critical effects on lyophilzation along with the distribution of these lyoprotectants on both sides of lipid bilayer.[16]

Biological Testing and Evaluation of Liposomes:

Liposomes are used as an important vehicle for drug delivery especially targeted delivery. Major conditions where these vehicles can be utilized are cancer and as antivirals. Stealth liposome can be used to extend the blood circulation time of encapsulated therapeutics A stealth liposome is a sphere-shaped vesicle with a membrane composed of phospholipid bilayer used to deliver drugs or genetic material into a cell.[17] A study by Luo et al.,[17] describes the drug release efficiency of Doxorubicin encapsulated porphyrin-phospholipid stealth liposomes. Near Infrared which can penetrate tissues is an intriguing external trigger for drug release and can be applied with precise spatial and temporal control, thereby help achieve light triggered drug delivery. On observing the results for this study, Luo et al. reported that Dox in stealth PoP liposomes had a long circulation half-life in mice of 21.9 hrs and was stable in storage for months. A single chemophototherapy treatment with Dox-loaded stealth PoP liposome (at 5-7 mg/kg Dox) eradicated tumors while corresponding chemo- or photodynamic therapies were ineffective. Thus, stealth liposome has shown tremendous potential in release of encapsulated drug in cancer therapy. Recently impressive therapeutic improvements were described with the use of corticosteroid loaded- liposome in experimental arthritic models.[18] A significant part of liposome drug delivery involves loading of drugs into the liposomes. This can be achieved passively (ie. The drug is encapsulated during liposome formation) or actively (ie. after liposome formation).[3,19] 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. Water soluble drugs that have protonizable amine functions can be actively entrapped by employing pH gradients. Some significant benefits of drug loading in liposomes include improved solubility of lipophilic and amphiphilic drugs, passive, and active targeting to the cells of immune system, site specific targeting, improved transfer of hydrophilic, charged molecules.[3]

Biological evaluation of liposomes includes monitoring the pharmacokinetics, in-vitro testing, and efficacy of the particular liposome formulation. A study by Wang[20]., describes the preparation and in-vitro evaluation of an acidic environment-responsive liposome for paclitaxel tumor targeting. The study uses acid sensitive material cholesteryl hemisuccinate (CHEMS) to increase the accumulation of drug in the tumor site. To determine the encapsulation efficiency and content of paclitaxel, High Performance Liquid Chromatography was used. Furthermore, paclitaxel-loaded acid sensitive liposomes were prepared by thin-film dispersion method. The particle size, polydispersity index and zeta potential were determined as well. The shape of liposomes was observed by Transmission Electron Microscopy (TEM). The in-vitro release characteristics were studied via dynamic dialysis method which resulted in the liposomes possessing acid sensitivity and sustained release properties.[20] In comparison to free paclitaxel, the paclitaxel-loaded acid sensitive liposomes showed a higher cytotoxicity and a better cellular uptake rate which posed them suitable for targeted cancer therapy with paclitaxel.

Microscopic imaging is an important aspect for the structural and morphological evaluation of liposomes. A study by S. Bibi et al.,[21] describes the use of different microscopic imaging techniques to evaluate the structural properties of liposomes. Larger vesicles such as multilamellar and giant unilamellar vesicles can be viewed using

light microscopy techniques. Some of the most popular techniques include light, fluorescence, confocal microscopy, and various electron microscopy techniques such as transmission, cryo, freeze fracture and environmental scanning electron microscopy.[21] In Transmission electron microscopy, sample preparation involves a small amount of hydrated specimen being placed onto a grid. A negative stain such as Uranyl Acetate or Osmium Tetroxide is used so that the vesicles can be viewed against the stained background. Fluorescent microscopy is used for tracking particulate delivery systems in biological environments and provide information regarding the structure of bilayer vesicles. It allows assessment of various parameters since probes can be placed in both the aqueous and bilayer compartments. A review by Klang et al.,[22] describes the electron microscopic techniques used for pharmaceutical systems. In Scanning Electron Microscopy, the image is formed point by point by scanning a focused electron beam across the surface of a solid specimen. One of the most advantageous characteristics of SEM analysis is the pronounced depth of focus in combination with the method of image formation: projecting areas cast shadows while recessed areas appear dark. Thus, the image allows the human eye to interpret and readily comprehend the obtained information.[22] Another advantage is that there is no sample preparation required for solid samples and large areas can be investigated with a high depth of focus. One major disadvantage of SEM is that data is collected one pixel after the other, which leads to longer exposure time to the electron beam.

II. Conclusion:

Liposomes are multifunctional drug carriers especially in treating diseases. Among the most common disease conditions, Cancer treatment remains at the forefront of almost every drug development and delivery project. Nowadays, a new kind of dual functional drug liposome has emerged as a promising drug delivery system. Dual-functional liposomes are referred to the drug- containing phospholipid bilayer vesicles that possess a dual-function of providing the efficacy of drug and the extended effect of the drug carrier. Prospective studies need to be carried out on the biocompatibility, circulation rate and toxicity parameters of this liposome system. Additionally, combination drug therapy in liposomal formulations has significant advantages and tremendous potential of being a preferred drug delivery strategy compared to the conventional drug therapy.

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