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LIPOSOMES: REVOLUTIONISING FOR DIAGNOSIS & MANAGEMENT OF ATHEROSCLEROSIS

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ABSTRACT

Atherosclerosis is one of the principal contributors to cardiovascular disease (CVD) and is a chronic disease characterized by the accumulation of lipid plaques and immune cells within the walls of arteries, leading to vascular inflammation, decreased blood flow to critical tissue regions and increased risk of heart attack and stroke. It is increasingly becoming a major health concern globally due to poor lifestyle and continues being a leading cause of morbidity and mortality. Currently available therapeutic strategies largely depend on the usage of conventional dosage forms, which present with a host of issues like non-specific drug distribution, enhanced drug toxicity and adverse effects. Liposomes are artificial lipid bilayer vesicles with an aqueous core. They have emerged as promising alternatives, gaining immense traction in recent decades for formulation of multitude of drugs from various therapeutic classes due its ability to bypass the limitations of conventional dosage forms through the provision for targeted delivery via surface modification with minimal toxicity. Liposomes are the ideal carriers for diagnostic and therapeutic purposes in a plethora of diseases, including atherosclerosis. They can be loaded with contrast agents to enable crisp, high resolution diagnostic images. Liposomes also exhibit promise in aiding the pharmacotherapy of atherosclerosis, chiefly owing to their ability to encapsulate both hydrophilic and lipophilic therapeutic agents and deliver them directly to atherosclerotic lesions, thereby improving accumulation and penetration. This review explores the numerous advantages and applications of liposomal technology, majorly highlighting their employment in the therapy of atherosclerosis. Future advancements in liposomal systems bear the potential to revolutionize the treatment of CVD and offer novel therapeutic strategies.

KEYWORDS: Liposomes, Cardiovascular diseases, Atherosclerosis, Treatment, Management, Applications.

INTRODUCTION

Cardiovascular diseases (CVDs), comprises of many conditions affecting the circulatory system including cardiac and vascular diseases. It mainly encompasses atherosclerosis and its subtypes; coronary, cerebral and periphery. The major complications include but are not limited to myocardial infarction, ischemic stroke, heart failure, cardiac valvopathies, arrhythmia.[1] They remain the leading cause of mortality worldwide and chiefly contribute to decreased quality of life and increased healthcare costs. The total number cases drastically doubled from 271 million in 1990 to a staggering 523 million in 2019. The number of CVD deaths also increased from 12.1 million in 1990 to 18.6 million in 2019.[2]

ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory disease affecting elastic and musculoelastic arteries and is associated with the formation of atheromatous plaques, mainly of LDL cholesterol.[3] The prolonged build-up of such plaques in the subendothelial intimal layer of large- and medium-sized arteries causes vessel occlusion, which leads to significant stenosis that restricts blood flow and causes critical tissue hypoxia.[4]

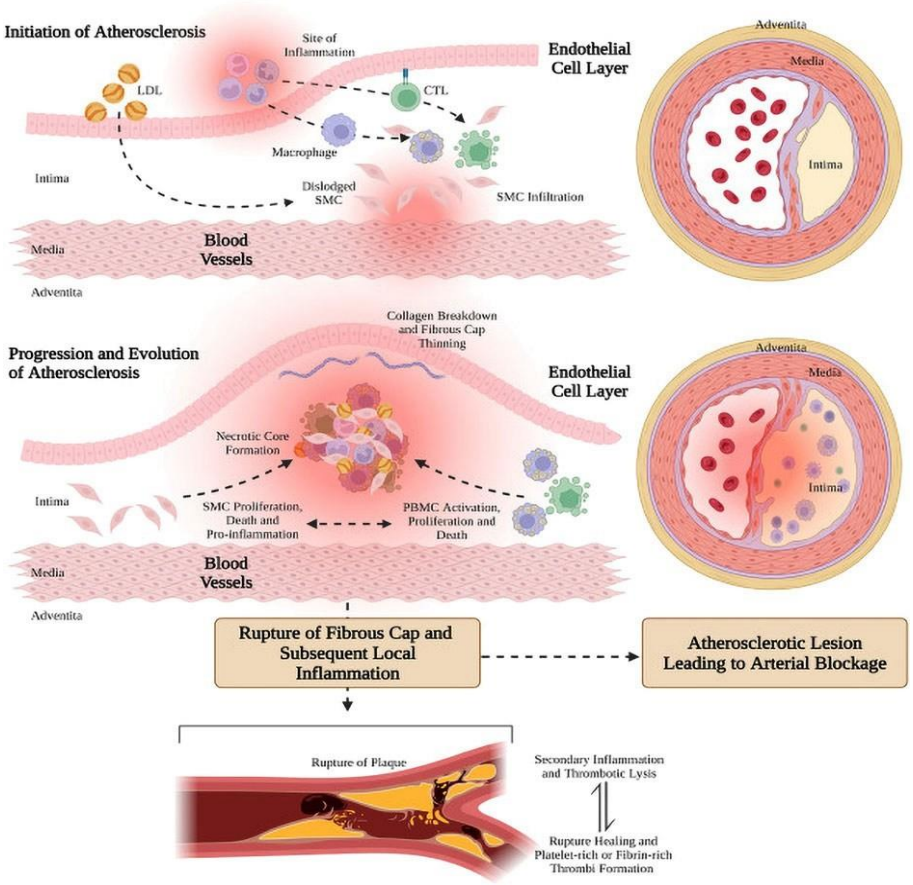

Figure 1: Pathogenesis, progression, and evolution of arterial atherosclerotic plaque.[5]

The conventional pharmacological treatment route indicates the administration of drugs involved in lowering lipid levels and anti-thrombotic agents that prevent platelet aggregation and inhibit thrombus formation.[6] Anti-inflammatory drugs are also gaining recognition as a potential therapeutic option in the treatment of atherosclerosis, but require extensive trials and detailed, conclusive research.[7, 8, 9]

Statins target hepatocytes and inhibit HMG-CoA reductase, the enzyme that converts HMG- CoA into mevalonic acid, a cholesterol precursor. They alter the conformation of the enzyme and prevents HMG-CoA reductase from attaining a functional structure. Statins also hinder the hepatic synthesis of apolipoprotein B100, producing a reduction of the synthesis and secretion of triglyceride rich lipoproteins.[10]

Patient's response to statins show considerable variability, with decline in LDL-C levels following the administration of statins ranging from 5% to 70%. However, some patients present as statin resistant. Alternatives to statin therapy include use of cholesterol absorption inhibitors and bile-acid sequestrants; yet, they lack the equivalent potency of statins in their ability to reduce LDL-C levels.[11]

As a result, statins remain the mainstay of atherosclerotic treatment, but they do not come unattached without grave side effects, the two major being muscular and hepatic AEs.[12]

Statin associated muscle symptoms (SAMS) are the most prominent which manifest as myositis, myalgia, myopathy with or without creatine kinase elevation. It may also present as rhabdomyolysis in certain critical cases.[13]

Elevation of liver enzymes (in particular, alanine and aspartate transaminases [ALT and AST]) is another well-recognized adverse effect of statins.[12, 14] Another described clinical manifestation of statin-induced liver injury (SILI) is the drug-induced autoimmune hepatitis mainly demonstrated with atorvastatin but also with other statins.[15,16]

Numerous clinical trials also claim that statins contribute in increasing the risk of onset of Type 2 Diabetes Mellitus.[17,18,19]

Critical gaps in current CVD treatment include the inability to detect and target specific pathological pathways, such as inflammation, thrombosis or proliferation within the heart or blood vessels, without adversely impacting healthy tissues. Nanomedicine proposes excellent potential for tackling of site-specific processes without the burden of systemic adverse effects. Moreover, it enables platform nanotechnologies that

possess versatile diagnostic and/or therapeutic benefits and 'all-in-one' type theragnostic.[20]

INTRODUCTION TO LIPOSOMES

The introduction of novel drug delivery systems is considered a promising mechanism to boost drug bioavailability and improve the ease of administration. The incline towards liposomal formulations in recent times by the pharmaceutical industry and healthcare practitioners alike is due to its multitude of benefits for the human body.

Liposomes can be simply defined as spherical vesicles having an aqueous core enclosed by one or more phospholipid bilayers or lamellae.[21] The name liposome is derived from two Greek words: 'Lipos' meaning fat and 'Soma' denoting body. Bangham et al in 1964 were the first to describe liposomes during their experimentation on blood clotting, where they illuminated the formation of bio membrane systems upon mixing water with phospholipids. Due to their morphological similarity to biological cell membranes, they established the foundation for a widely used drug delivery system, suitable for both hydrophobic and hydrophilic drugs.[22]

Membranes are usually comprised of phospholipids, which are molecules possessing a head group and a tail group. The head is attracted to water i.e. hydrophilic and the long hydrocarbon chain comprising the tail is hydrophobic. In the presence of water, due to their inherent hydrophilicity, the heads line up to form a layer facing the aqueous interface. The tails are repelled by water, and incline inwards to form a surface away from the interface. This characteristic lipid bilayer is seen in all the cells of the human body.[23] Liposomes possess phospholipid bilayer that bears identical structural characteristics and properties to biological membranes, implying a major benefit in membrane compatibility due to their amphiphilic properties.

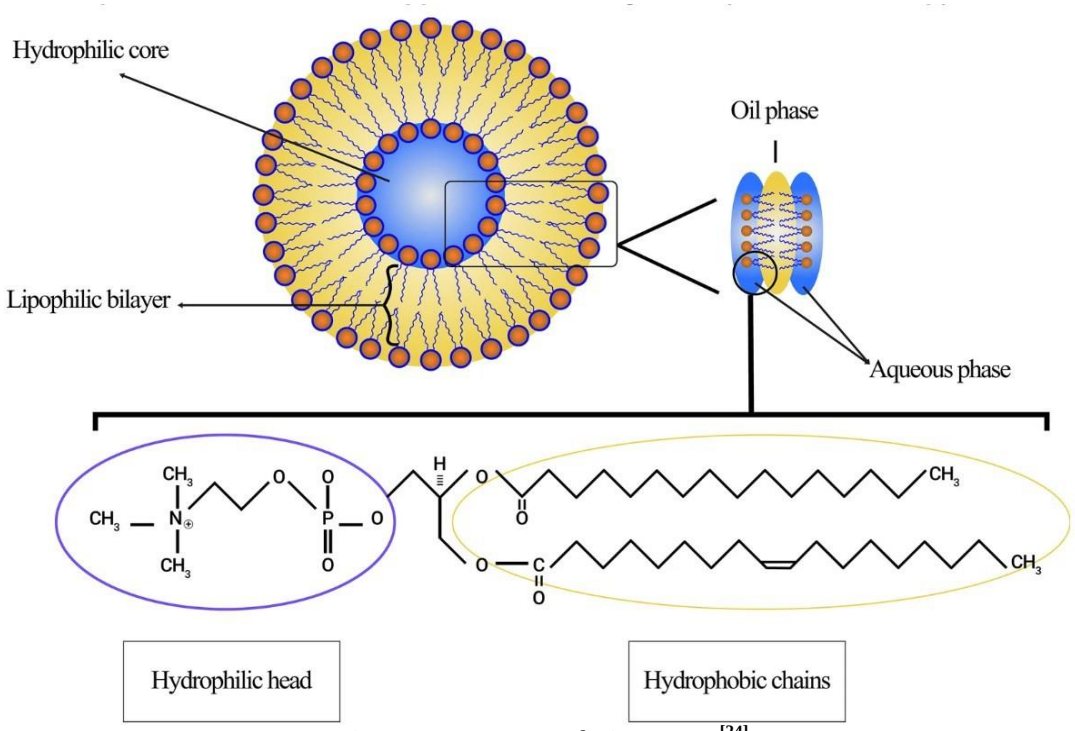

Figure 2: Structure of Liposomes.[24]

Liposomes deliver a plethora of advantages over conventional dosage forms[25, 26]

1. Increased bioavailability compared to oral route.
2. Ability to incorporate both hydrophilic and hydrophobic compounds.
3. Increased drug loading capacity.
4. Facilitates encapsulation of drugs sensitive to GIT environment and metabolic enzymes i.e. provides a protective effect and improves drugs stability.
5. Prolonged half-life of drugs and enhanced drug permeation due to lipid bilayer and ability to achieve sustained release products.
6. Improved biocompatibility and reduced toxicity of drug.
7. Non-invasive and hence reduce patient discomfort and contamination risk.
8. Possibility of surface modification to achieve targeted therapy and improved pharmacokinetic parameters.

Contrarily, they also present with a number of limitations[25, 26]

1. High production cost
2. Threat of leakage or fusion of encapsulated drug molecules
3. Rapid uptake by RES
4. Risk of dose dumping

5. Batch to batch variation

COMPOSITION OF LIPOSOMES

Liposomes are primarily composed of a lipid bilayer encompassing an aqueous core. The primitive liposomal formulations were comprised exclusively of natural lipids; presently, they can include both natural and/or synthetic phospholipids and surface active agents. They boast the capability of entrapping both lipophilic and hydrophilic drugs, in the lipid bilayer and the aqueous core, respectively.[27]

Phospholipids: Natural phospholipid are defined as the phospholipids isolated from natural sources like egg yolk, soybean, rapeseed, and sunflower seed. Through the process of hydrogenation, natural phospholipids may be transformed to saturated phospholipids, which are also naturally occurring or they may be exposed to enzymes to partially remove fatty acids (phospholipase A2) or to convert a polar head group (phospholipase D).[28]

Synthetic phospholipids can be created largely through the modification of the non-polar and polar regions of natural phospholipid molecules. Examples of widely used synthetic lipids are dipalmitoyl phosphatidylcholine (DPPC), di-myristoyl phosphatidylcholine (DMPC), di-stearoyl phosphatidylcholine (DSPC) and hydrogenated soy phosphatidylcholine (HSPC).[28, 29]

Based on the alcohol groups present in their chemical structures, phospholipids are differentiated into glycerophospholipids, and sphingomyelins.[30] Phosphatidylcholine (PC) molecule represents the most commonly occurring phospholipid. It is water insoluble and in aqueous media, aligns itself to form planar bilayer sheets to attenuate the undesirable action between the bulk aqueous phase and the lipophilic hydrocarbon chains.[31] Examples of other phospholipids that are the derivatives of phosphatidic acid include.

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

The liposomal stability is dependent on degree of unsaturation, fatty acid side chains and phase transition temperature of phospholipids.[31] The phase transition temperature of phospholipids is defined as the temperature at which the phospholipid's physical state converts from gel phase to liquid crystalline phase.[32] During the transition from the more ordered gel phase to the less ordered liquid-crystalline stage, drug molecules experience less resistance when moving across the bilayer, displaying an increase in rate of permeation, which peaks near T_m. Hence, nano formulations comprised of lipids having characteristic high T_m, e.g: dipalmitoyl phosphatidylcholine (DPPC) or distearoylphosphatidylcholine (DSPC), illustrate a rigid and highly stable lipid bilayer along with diminished leakage of the encapsulated drugs.[33, 34] Glycerophospholipids such as di-myristoyl, dipalmitoyl, or di- stearoyl PC are produced due to the variation in length of the nonpolar groups.[35]

Steroids: The structural properties of liposomes are largely affected by cholesterol. When it is incorporated into liposomal formulation, it induces a dense packing of phospholipids and inhibits their transfer to high-density lipoprotein (HDL) and low-density lipoprotein (LDL). In fact, cholesterol is a hydrophobic molecule and preferentially interacts with the core of the membrane, thus stabilizing it.[27] Overall, this results in a decrease of the fluidity and water permeability of liposomes, while the bilayer is less inclined to penetration by foreign substances. The enhanced mechanical rigidity of liposomes confers extra stability and prevents interaction with protein elements such as transferrin, albumin, and high-density lipoproteins, which may contribute to the loss of activity and efficacy.[36, 37]

Surfactants: The drug release properties and encapsulation efficiency can be modified through the utilization of surfactants. They work by the mechanism of reducing surface tension between two immiscible phases.[38] When a surfactant is used in higher concentration, it saturates the liposome surface and hence prevent them from aggregating.[39] Due to the hydrophilic characteristics of the surfactant head portion, a reduction in vesicle size was also reported which was attributed to the shorter length of the hydrophobic backbone relative to the hydrophilic head.[40]

CLASSIFICATION OF LIPOSOMES

Based on the particle size and number of layers, liposomes can be classified into the following categories; unilamellar vesicles (SUV and LUV), oligolamellar vesicles (OLV), multilamellar vesicle (MLV), and multivesicular vesicles (MVV). Additionally, UVs can be further classified into small unilamellar vesicles (SUVs) with a particle size of <100 nm, large unilamellar vesicles (LUVs) with a particle size ranging between 100 and 1000 nm, and giant unilamellar vesicles (GUVs) with a particle size > 1 µm according to their size.[41]

In a unilamellar structure, liposomes constitute a single phospholipid bilayer. On the contrary, a number of phospholipid layers which are about 0.5-1.5 µm are noted in multilamellar vesicles. MVV consists of a multilamellar structural arrangement with concentric phospholipid spheres as many unilamellar vesicles are produced within larger liposomes.[42] [43]

Owing to their added benefit of longer circulation time and targeted drug delivery to disease site, SUV's are the most commonly found liposome in marketed formulations. Hence, they are the carriers of choice for

anti-cancer drugs and vaccine.[44] ULV are ideally preferred for incorporating hydrophilic drugs whereas MLV are suited for entrapping lipophilic agents due to their higher lipid content.[45]

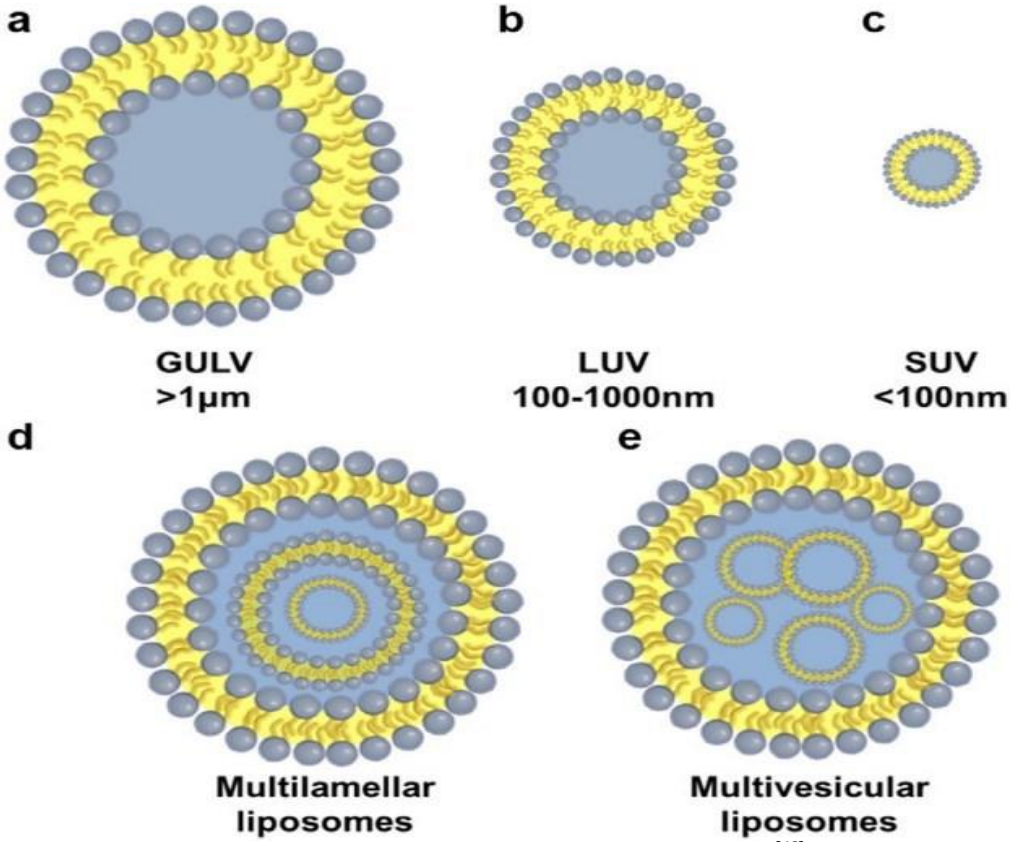

Figure 3: Classification of liposomes based on size.[46]

METHODS FOR PREPARATION OF LIPOSOMES

Liposome preparation methods may be classified into (a) bulk methods, which involves the transfer of phospholipids from organic phase to an aqueous phase, resulting in the formation of liposomes (b) film methods, in which lipid films are firstly coated on a substrate and thereafter hydrated to obtain liposomes. The techniques for liposome preparation can also be divided based on particle mean size, polydispersity and lamellarity of liposomes, primarily due to the challenge of control over these parameters with almost all preparation methods.[46] There are a few elementary steps involved in the preparation of liposomes which include.[35]

1. Evaporation of the organic solvent to obtain dried lipids
2. Formulating lipid dispersion in aqueous media
3. Purification of obtained liposomes
4. Analysis of the final preparation

The loading of drug into the liposomes can be achieved through two techniques.[43]

1. Passive loading techniques i.e. during liposome formation
2. Active loading techniques i.e. after liposome formation

The Passive Loading techniques further involve three methods.[43]

a. Mechanical dispersion methods
b. Solvent dispersion method
c. Detergent removal method

I. Mechanical Dispersion Method

Thin film hydration method (Bangham method)

In this technique, the phospholipids are firstly dissolved in an organic solvent such as chloroform or mixtures like chloroform and methanol. Then, the solvent removal step is undertaken by deposition of film under a vacuum. Once the complete evaporation of the solvent has been achieved, the phospholipid residue is hydrated using an aqueous buffer at a temperature above the transition temperature (Tm). The hydrophilic drug which is to be loaded into the liposomal aqueous core can be dissolved in the hydration solution. Subsequently, there is spontaneous swelling of the lipids due to hydration, contributing to the formation of liposome. The entire hydration process usually has a duration of 1-2 hours with the experimental temperature being maintained at 60–70 °C, or at temperatures above the phase-transition temperature of the phospholipid constituents. Stirring or mechanical agitation at this stage may assist to detach the lamellae of the swelling lipids from the vessel's internal surface.[35, 42, 47]

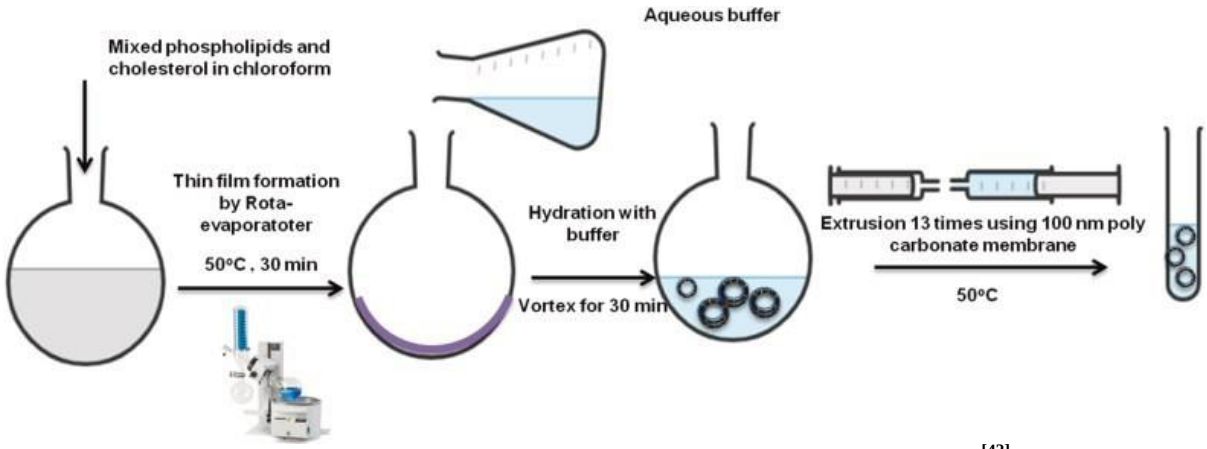

Figure 4: Thin Film Hydration Method for Preparation of liposomes.[42]

II. Solvent Dispersion methods

Ether Injection method

In this method, the lipids are dissolved in diethyl ether or a mixture of methanol and ether. The resulting mixture is then slowly injected to an aqueous solution of the drug to be encapsulated under low pressure or at a temperature of 55-65 °C.[35, 48] The synthesis of LUVs can be facilitated by the consecutive removal of the organic solvent under reduced pressure. This particular technique favours the formation of concentrated liposome solution with high entrapment efficiencies.[47]

The major pitfalls of this procedure are the non-uniformity in particle size of the obtained vesicles plus the exposure of the molecule to be encapsulated to elevated temperature, which can lead to undesirable effects on the safety and stability of the formulation.[49]

Ethanol Injection Method

In this technique, The phospholipids are in initially dissolved in ethanol and subsequently, injected rapidly to a pre-heated distilled water or buffer solution such as TRIS-HCl. Due to the dilution of ethanol in the aqueous solution below a critical concentration, the dissolved lipids undergo self-assembly in the aqueous phase.[50] Ethanol can be removed by evaporation under vacuum using a rotary evaporator, dialysis, or filtering. This methods produces SUVs and vesicles with higher polydispersity indexes.[22, 51] The suspension of liposomes is thereafter stirred at room temperature for approximately 15 minutes. This technique presents various advantages like rapidness, ease of performance, and reproducibility. Furthermore, it prevents oxidative alterations or phospholipid degradation and can result in production of SUVs without extrusion or sonication.[52]

III. Detergent removal and dialysis method

When unilamellar liposomes are required, detergent removal process for liposome preparation is superior over other methods.[53] Here, phospholipids and high CMC surfactant are dissolved in suitable organic solvent. When detergents are utilized at their CMC, they can solubilize lipids. As the detergent is detached, the micelles progressively aggregate to form LUVs. Removal of the detergent can be achieved through dialysis.[43,54,55] However, a major disadvantage of this process is that additional excipients are avoided due to impurities risk and elevated preparation costs.[53]

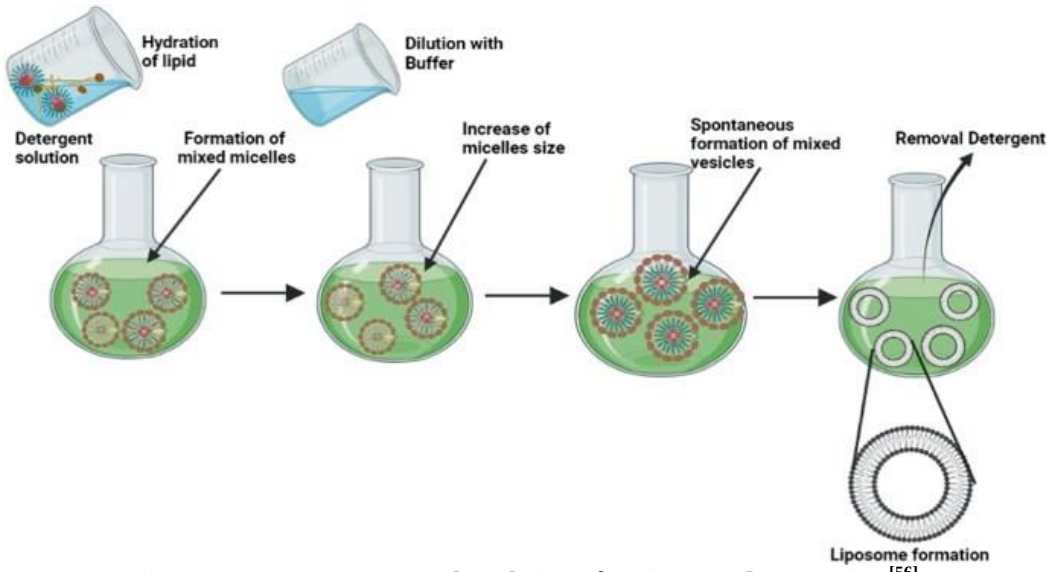

Figure 5: Detergent Removal Technique for Liposomal Formation.[56]

Dehydration-Rehydration Method

This method has a prime benefit of being organic solvent free and is utilized in the production of LUVs through sonication. It is based on the dispersion of the lipids at low concentrations into an aqueous drug solution and this mixture is then subjected to sonication. A dehydration step is then undertaken to evaporate the aqueous phase under nitrogen, which leads to the formation of multilayered film entrapping the drug molecules. Subsequently, a hydration step is carried out to form large vesicles encapsulating the drug molecules.[57] Dehydration/rehydration technique can be used to enhance and optimize the encapsulation efficiency and the in-vitro stability of macrolide and aminoglycoside antibiotic liposomes.[58]

Microfluidic Technology

Microfluidics is a technology that facilitates precise control and manipulation of fluids and fluid interfaces at the micrometer scale.[59] As illustrated below, this technique involves the dissolution of lipids in ethanol or isopropanol solvent and then successive propulsion within microscopic channels, ranging from 5-500 µm cross-sectional area.[60, 61]

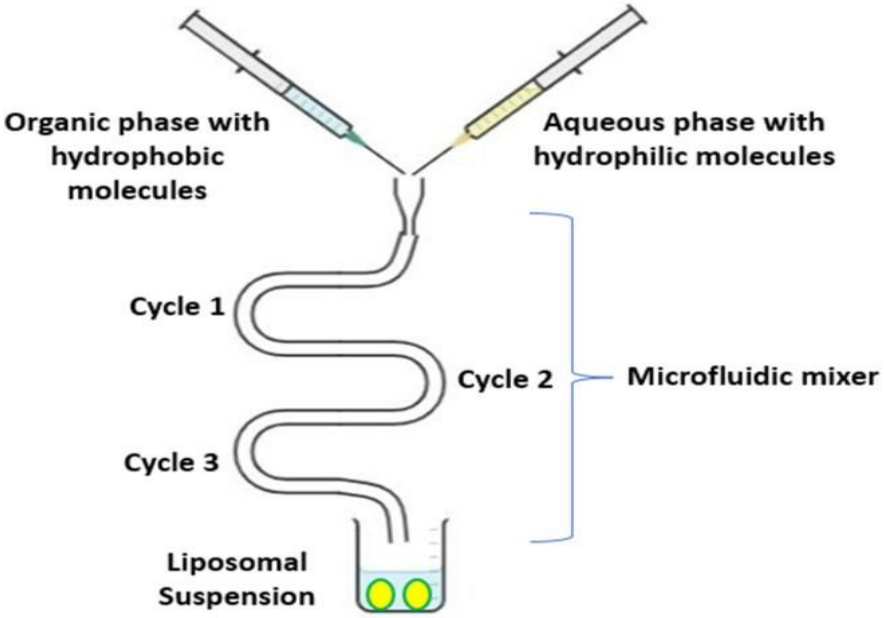

Figure 6: Microfluidic Methodology for synthesizing liposomes.[42]

Droplet based microfluidic systems can be used to produce giant liposomes.[62,63] Through this technique, two immiscible aqueous and oil phases are allowed to flow through a microchannel, leading to the formation of uniform small droplets of one phase. For the production of small vesicles, micro-hydrodynamic focusing (MHF) has been reported. It was developed by Jahn et al. and culminates in the formation of homogenous SUVs and LUVS whose particle size ranges from 40 to 140 nm.[61] Another technique used for the production of giant vesicles is the pulsed jet flow method. It involves drying a phospholipid solution in micro-capillaries. The subsequently formed lipid film is hydrated with the help of a perfusion process, resulting in the formation of giant vesicles with enhanced encapsulation efficiency.[64] The proven advantages of this microfluidic methods are the precision of mixing and control over the fluid flow rate, and hence allows for reproducible control of particle size and size distribution.[59]

Supercritical Fluid Technology

Supercritical fluids (SCFs) are those that bear the desirable properties of both liquids and gases. For instance, a small change in pressure or temperature leads to large changes in SCF density and solubility of various compounds in the SCF. The use of SCFs is rapidly increasing due to their ability to facilitate efficient separation and purification compared to other organic solvents.[22] CO_2 is the most commonly used gas which becomes supercritical at its critical temperature (T_C) of 31.1°C and critical pressure (P_C) of 73.8 bar.[65]

The general supercritical fluid method consist of two stages.[66,67] Initially the lipids are dissolved in supercritical CO_2 is performed under high pressure (P=250 Bar). Following this step, the supercritical homogeneous solution produced is subjected to expansion at T = 60°C, accompanied with the addition of a small amount (approximately 7% *v*/*v*) of ethanol. Using this approach, the encapsulation efficiency attained is 15% and total amount of ethanol is 15-times lower compared to the ethanol injection method.[69] The expanded liquid is then mixed thoroughly and with the aid of a nozzle, is injected into an aqueous phase, leading to the production of liposomes containing encapsulated hydrophilic drugs.[66]

The various techniques involved use of supercritical

fluids are.[47]

1. Supercritical Reverse-Phase Evaporation (SC-RPE) Method
2. Supercritical Anti-Solvent (SAS) Method
3. Rapid Expansion of a Supercritical Solution (RESS) Method
4. Supercritical-Assisted Liposome Formation (Super Lip) Method
5. Depressurization of an Expanded Liquid Organic Solution into Aqueous Suspension (DELOS) Method

EVALUATION OF LIPOSOMES

Physical and chemical parameters need to be monitored, so that we can assure that the preparation of liposomes is reproducible and that it has the desired function. The characterization of liposomes should ideally be done immediately after preparation and throughout its shelf life to ensure the adequate quality control of the product. The methods adopted should be precise and rapid. Dynamic light scattering (DLS), high performance liquid chromatography (HPLC), size exclusion chromatography (SEC), field flow fractionation (FFF), fluorescence spectroscopy, and nuclear magnet resonance (NMR) are bulk methods for liposome analysis.[70]

Particle size and size distribution

The pharmacokinetic pathway of liposomes in the body, such as systemic circulation and the clearance by MPS, the extravasation into tissue interstitial, interstitial transport in the extracellular matrix, and cellular uptake and intracellular trafficking, are dimension-dependent.[71] It is especially an important parameter for formulations intended for inhalational or parenteral route.[72, 73, 74] The mean hydrodynamic radius (Rh) and polydispersity index of a dispersion can be measured accurately using nanoparticle tracking analysis, dynamic light scattering, and tunable resistive pulse sensing.[75]

A number of techniques, including microscopic, hydrodynamic, and diffraction light scattering methods, are employed to determine the particle size and size distribution. Microscopic techniques include optical microscopy, scanning electron microscopy (SEM), negative stain TEM, and freeze-fracture TEM which can be utilized to obtain imaging and detailed information about thickness of the bilayer and inter-bilayer distance of liposomes. Hydrodynamic techniques such as ultracentrifugation, field flow fractionation, gel exclusion chromatography, and analytical centrifugation procedures are adopted to estimate the molecular mass and polydispersity index. Diffraction light scattering techniques which mainly encompass laser light scattering, quasi-elastic light scattering, and photon correlation spectroscopy offer insights into the size of the lipid vesicles. These techniques can be used to estimate the mean diameter.[76,77] Fluorescence microscopy can also be used to measure the size and lamellarity of fluorescently labeled liposomes.[70]

Surface Charge

The zeta potential of the liposome is defined as the complete charge which is obtained by the liposome in a medium. It may be positive, negative, or neutral.[78,79]

Zeta potential majorly influences the cellular uptake of liposomes and targeted drug delivery. To measure the zeta potential of the liposomal dispersion, Laser Doppler electrophoresis and Zeta sizer are utilized. Their main mechanism involves the application of an electric field on the particles and based on the scattering of incident laser on the moving particles, the charge can be determined. DLS is also commonly used to estimate the zeta potential. Factors such as pH, temperature, ionic strength, and particle concentration can have a significant effect on the zeta potential. the lipid composition, specifically the positive and negative surface charges of the phospholipids utilized in the liposomes can affect the overall surface charge of the liposomal product and blood circulation time of the formulation.[80] Liposomal suspensions possessing zeta potential higher than 30 mV or lesser than -30 mV are considered stable, chiefly because at this potential, the particle remains distant and do not undergo aggregation.[81]

When a charged liposome is introduced into an external electric field, E0, due to the influence of the resulting electrostatic force, it is mobilized immediately. During this, it also drags part of the diffuse double layer with it. The velocity of the resulting particle movement is proportional to the E0 value and to the particle mobility. The measured mobility attains a constant value after some time, which is mainly attributed to the balance of forces between the driving electrical force and the resistance offered by the medium. The zeta potential can then be calculated with Smoluchovsky Henry equation.[82]

$$\upsilon E = 4\pi\varepsilon 0\varepsilon r \frac{\zeta}{6\pi\mu}(1+\kappa r)$$

Encapsulation Efficiency

The encapsulation efficiency can be defined as the proportion of the aqueous phase containing the hydrophilic drug that is captured during the creation of liposomes. It is indicated as % entrapment/mg of lipid. The bioavailability of drug is directly proportional to the entrapment efficiency and hence an increase it the encapsulation efficiency will significantly improve the biopharmaceutical characteristics of drug.[83]

For estimation of the encapsulation efficiency, the free drug needs to be separated from the liposomal formulation, either by dialysis, gel filtration, or centrifugation. However, these techniques may prompt liposome destruction and trigger the release of the encapsulated drug. Dialysis is the considerably gentle, but is accompanied with the drawbacks of being time-intensive and instrumentally challenging.[41, 84]

Gel filtration and centrifugation are thus the preferred techniques. Once the encapsulated and free drug undergo separation, the liposomal membrane is disrupted and the quantification of released encapsulated material is ascertained by fluorescence spectroscopy, enzymatic, or electrochemical methods.[85]

The purification and separation of liposomes can be performed by mini-column centrifugation method and protamine aggregation method is employed for liposomes with neutral and negatively charge.[86]

Lamellarity Determination

The number of lipid bilayers present in the liposome is referred to as the lamellarity. This property influences the encapsulation efficiency, drug release kinetics, the biopharmaceutic parameters of the drug in the body and the potential applications of liposomes.[87]

A multitude of techniques such as ^{31}P-nuclear magnetic resonance (NMR), small angle X- ray scattering (SAXS) techniques and fluorescence spectroscopy are accepted as means to measure liposomal lamellarity. They provide insights into the size, uniformity, and lamellarity of liposomes.[85, 88, 89]

At the singular level, imaging techniques such as cryo-electron microscopy, freeze-fracture electron microscopy and light microscopy can be used to determine the lamellarity of single liposomes.[90, 91]

In-vitro Drug Release Studies:

Drug release is regarded as a dual-step procedure majorly involving the separation of the encapsulated drug from the medium containing the free/released drug, followed by the quantification of the released drug. The separation technique should preserve the carrier integrity and have no influence on the concentration equilibrium between the encapsulated and free drug. When drug release studied are carried out in simple media, the analytical methods used are adaptations of conventional bioanalytical techniques utilized for purification of nano-formulations. These include chromatographic methods, liquid-liquid extraction, and equilibrium methods.[92]

In vitro drug release studies are performed at 37°C using in vitro diffusion cells or dialysis bags. The diffusion cells or dialysis bags are submerged in receptor medium containing pH 7.4 buffer solution and agitated continuously to imitate in vivo environment. Periodically, the medium is collected, and the analysis of drug concentration in it is determined using HPLC and UV–Visible spectrophotometry. Simultaneously, a corresponding volume of fresh medium is added to the receptor medium.[93]

In-vivo Performance

The vesicular pharmacokinetic properties may influence the in vivo performance of drug- containing liposomes. The in vivo performance can be analyzed by intravenous administration of the liposomes, which discloses rapid hepatic and splenic clearance. Large liposomes with a particle size greater than 0.5 µm undergo phagocytosis, whereas smaller particles with size less than 0.1 µm are taken up by the liver parenchymal cells.[94]

Stability Studies

The shelf life of liposomes can be characterized by thorough stability studies. These involve physical stability, chemical stability, and biological stability. In physical stability, discoloration or change in colour of liposomes can be visually observed or deduced using TEM and AFM. Chemical stability consists of hydrolysis, oxidation and drug degradation that can be identified through the use of HPLC, TLC and HPTLC and thiobarbituric acid (TBA) test.[86]

APPLICATIONS OF LIPOSOMES

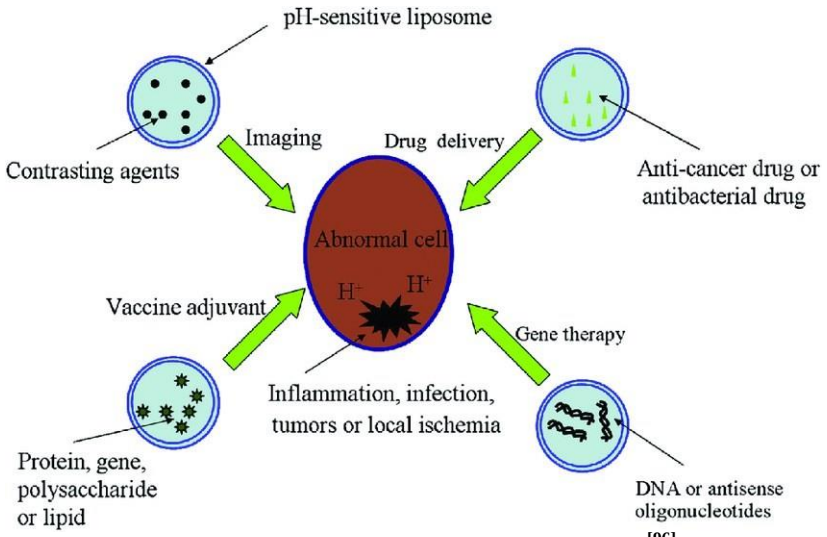

Figure 7: Clinical applications of liposomes.[96]

The encapsulation of drugs in liposome systems modifies the spatial and temporal distribution of the drug molecules in the body, vastly reducing the undesirable adverse toxic effects and enhancing the efficacy of treatment. Applications of liposomes in pharmacology and medicine can be divided into therapeutic and diagnostic applications of liposomes based on the material encapsulated which may include drugs or a variety of other diagnostic elements.[95]

BIOMEDICAL APPLICATIONS OF LIPOSOMES

Liposomes have a characteristic advantage of the ability to encapsulate both hydrophilic and hydrophobic drugs.[97] They are also biodegradable and relatively non-toxic compared to conventional formulation[98], making them suitable for a wide range of biomedical applications.

Protection Against Enzymatic Degradation of Drugs

The lipids utilized in liposome systems are resistant to enzymatic degradation, ensuring the protection of the entrapped drug as the lipid vesicles circulate in the extracellular fluid. Upon cellular entry, the encapsulated material is released through diffusion, shell dissolution, or shell degradation mediated by lysosomal enzymes. This attribute makes liposomes suitable for incorporating β-lactamase sensitive antibiotics such as penicillin and cephalosporins to protect them from the β-lactamase enzyme. Liposomes also provide protection for encapsulated drugs in the gastrointestinal tract environment and aid in the gastrointestinal transport of various compounds.[99]

Vaccine Delivery

Liposomes propose several benefits when selected as carriers for vaccine agents. They are biodegradable and non-toxic. Drugs integrated into liposomes can stimulate both humoral immunity after oral administration as well as triggering cell-mediated immunity. Liposomes are currently being harnessed for the delivery of oral vaccines in various immunization procedures. After 25 years since the discovery of the immunological adjuvant properties of liposomes, they are now formally recognized as the primary candidate for the base of an oral vaccine against hepatitis A, which is undergoing licensing for human use.[100]

Drug Targeting

The concept of "drug targeting" refers to the strategy involving drug delivery directly to the specific or intended site of action, which may be an organ, a certain cell population or even a region within a cell. This bypasses the issue of non-specific systemic circulation and distribution to all the body tissues in varying degrees which is inefficient and results in the manifestation of toxic side effects. Conversely, limiting the drug's distribution to the specific target site should increase effectiveness at lower doses while simultaneously reducing toxicity. Therefore, the advantages of drug targeting include the minimization of drug wastage and adverse effects and the capability to deliver a drug to a biological region that may not be typically accessible to the free or untargeted drug.[101] The method of targeting drugs using liposomes chiefly involves attaching ligands (such as antibodies, sugar residues, apoproteins, or hormones) to the lipid vesicles. These ligands recognize specific receptor sites, causing the liposomes to accumulate at these target sites. This approach helps to avoid or minimize the preferential distribution of liposomes to the reticuloendothelial system (RES), which includes the liver, spleen, and bone marrow and prevent it is premature clearance. The selection of a ligand is primarily based on its ability to be recognized by and its specificity for the target site. For instance, in cancer treatment the drug can be targeted to malignant tumour cells using receptor-specific ligands, which could be specific antibodies for antigens produced by the tumour cells. Hence, this requires the pre-requisite knowledge and identification of the antigens produced by the tumour cells. Additionally, molecules with oligosaccharide chains have been utilized as ligands for guiding and attaching specifically to ganglion sites in cells.[102]

Topical drug delivery

The effectiveness of liposomal drug delivery when applied to the skin surface has been well- established. Liposomes enhance the skin's permeability to various encapsulated drugs while reducing their side effects by allowing for lower doses.[103] Furthermore, liposomes have become essential in skin care cosmetics, where they are applied dermally in the form of a solution or in hydrogel formulation. Hydrophilic polymers serve as appropriate thickening agents for the gels. However, in some cases, the liposomes may become trapped in the polymeric network of the hydrogels which inversely affects their release rate and extent, thereby affecting their bioavailability into the skin.[104]

Enhanced antimicrobial efficacy/ safety

Antimicrobial agents have been encapsulated in liposomes for two reasons. Firstly, they extend protective effect to the entrapped drug against enzymatic degradation as is observed in the case of penicillin and cephalosporin antibiotics against the degradative action of β- lactamase, which is produced by certain microorganisms. Secondly, the lipophilic nature of the vesicles promotes enhanced cellular uptake of the antibiotics into the microorganisms, thereby minimizing the effective dose and the incidence of toxicity as is exemplified by the liposomal formulation of amphotericin B.[105]

APPLICATION OF LIPOSOMES IN ATHEROSCLEROSIS

Diagnostic applications of liposomes in atherosclerosis

In previous times, the diagnosis of atherosclerosis was restricted to a few methods such as assessment of electrocardiography at rest and during exercise, evaluation of the ankle- brachial index, and invasive angiography. In recent times, atherosclerotic plaque

visualisation is possible through non-invasive imaging techniques.[106] Plaque imaging techniques currently used clinically include ultrasound, magnetic resonance imaging (MRI), and computed tomography (CT), as well as nuclear imaging techniques, such as positron emission tomography (PET) and SPECT. Liposomes are a promising revenue as the utilisation of diagnostic agents for the non-invasive early detection of atherosclerosis. Liposomal imaging agents supply direct signals from the lesion site. This facilitates easier plaque detections and allows inferences to be made about its size and composition. The principal role of liposomes in the diagnosis of atherosclerosis is to behave as a carrier and delivery mechanism for contrast agents, thereby improving the resolution of the diagnostic image. The liposomes can be made multifunctional by the simultaneous loading of various different contrast agents. Functional measurements including peripheral artery tonometry, flow-mediated dilatation, and pulse wave velocity can be employed for the diagnosis of early endothelial dysfunction, which can further be visualized using PET and CT scans. More progressive lesions with lipid accumulation can be observed using methods like MRI, coronary CT angiography, coronary intravascular ultrasound.[107]

Nuclear imaging (PET and SPECT)

Nuclear imaging techniques rely upon a radiation source present in-vivo. Both SPECT and PET techniques construct images based upon the detection of gamma rays emitting from radioactive substances within tissues. 18 F-fluorodeoxyglucose (FDG) is a radio-labelled glucose analogue that has possible application in PET scanning. 18 F-sodium fluoride is another PET radiotracer that is used for dynamic evaluation of coronary microcalcification. In reference to atherosclerotic diagnosis, this technique is beneficial in identifying macrophages and inflammation markers.[108] SPECT can also be utilised with a variety of tracers to detect inflammation and its hallmarks.[109] Currently under investigation, liposome-based probes serve as positive prospects to enhance SPECT and PET imaging in a range of diagnostic settings. For example, PS-containing liposomes of 100 or 200 nm (PS100 and PS200) were injected into Watanabe heritable hyperlipidaemic rabbits, scanned with SPECT and compared with CT images 48 h after injection.[110]

Computed Tomography (CT) Scan

CT Scan is an X-ray-based imaging technique that is rapid and comparatively inexpensive. However, in this diagnosis technique, a bolus injection of contrast agent is essential. CT angiography is suitable for the detection of calcification in atherosclerosis. With CT, liposomes have a similar role to that in MRI. Liposomes (either simple or PEGylated) typically carry a CT contrast agent, such as iopromide, gold, or bismuth.[111–114] Danila et al. encapsulated a contrast agent called 5 - [N-acetyl (2, 3-dihydroxypropyl) - amino) - N, N – bis (2, 3 – di hydroxypropyl) 2, 4, 6-triiodo-benzene - 1, 3 dicarboxamide (iohexol) into PEGylated liposomes with the aim of overcoming the short residence time and renal toxicity of free iohexol. The liposomes comprised dipalmitoyl phosphatidylcholine (DPPC), cholesterol, and a linker (molar ratio of 3:1:0.3) and were then conjugated to an antibody against ICAM-1 to target the plaque.[115]

Magnetic Resonance Imaging (MRI):

MRI is a useful approach for the detection of various plaque components, including the fibrous cap and lipid core. It provides detailed insights on plaque size, composition, endothelial permeability, and plaque neovascularization, as well as 3D images of the plaque at near-cellular resolution. Thus, for the detection of macrophages and macrophage-rich areas in atherosclerotic plaques, MRI is an invaluable tool. Targeting liposomes to the plaque enables increased accumulation of loaded contrast agent at the diagnostic site, which results in improved signal strength. An example of liposomes that contain an MRI contrast agent is PEGylated liposome into which gadopentetate Di meglumine (Gd-DTPA) has been incorporated.[116, 117]

As mentioned earlier, PS-enriched liposomes are recognized by macrophages located in plaques. Thus, loading gadolinium into such liposomes increases its concentration at the site of the plaque.[118] The mechanism responsible for the accumulation of gadolinium loaded liposomes in plaque has been attributed to ERR i.e. endoplasmic reticulum retention receptor.[119] Gadolinium has also been loaded into liposomes functionalized with antibodies against LOX-1 receptors in the dysfunctional endothelium of plaque.[120]

Ultrasonography

Ultrasound imaging techniques are beneficial for the detection of vulnerable atherosclerotic plaques. Using ultrasound, it is possible to perform a catheter-based, real-time measurement of carotid intima-media thickness. Emerging techniques, such as intravascular photoacoustic-ultrasound (IVPA-US) imaging, can provide more precise information concerning the morphology of the arterial wall. The major advantage of IVPA-US over conventional ultrasound methods is that it can provide information on the composition of the plaque.[121, 122] The layered structure of liposomes allows for the entrapment of gas bubbles, which efficiently reflect sound waves and produce ‘acoustically reflective’ liposomes. Such acoustically reflective liposomes can be attached to antibodies (anti-fibrinogen or anti- ICAM-1) to enable the recognition and targeting of plaque. Multilamellar acoustic liposomes have been prepared that comprise phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and cholesterol, with entrapped gas bubbles between the lipid layers. This resulted in a significant acoustic enhancement during ultrasound imaging when evaluated in the Yucatan mini-swine model of induced atherosclerosis.[123, 124] An alternative approach to the

IVPA-US method is to include a contrast agent, such as indocyanine green (ICG) J-aggregate (IJA), into liposomes. Such liposomes have been targeted towards FRb, which is overexpressed on activated macrophages in atheromatous plaque. Increased uptake of FRb-targeted liposomes was evident in-vivo using an ApoE mouse model of atherosclerosis.[125]

THERAPEUTIC APPLICATIONS OF LIPOSOMES IN ATHEROSCLEROSIS:

Liposomes bear a multi-layered structure that permits the use of a single liposomal formulation as a drug delivery system. They are the ideal carriers for the incorporation of genes, stem cells, anti-inflammatory and antiangiogenic drugs to the desired site of plaque formation. Liposomes have also been documented to reduce the levels of LDL cholesterol and been employed in vaccine development, targeting atherosclerotic mediators.[126]

For the purpose of demonstrating the critical function of activated platelets in pathogenesis of atherosclerosis, the progression of atherosclerotic lesions, and occurrence of thrombosis in vascular disorders, Srinivasan et al. attached ligands on activated platelets, that bind to surface receptors (e.g., P-selectin and integrin GP IIb/IIIa) and liposomes.[127]

Presently, pharmacotherapy options for atherosclerosis predominantly concentrate on either reducing excessive cholesterol levels, or managing inflammation to inhibit the progression of the disease. Yet, they are incapable of directly dissolving atherosclerotic plaques and reversing atherosclerosis, which can be contributed to the poor accumulation of drug molecules in the plaques.[128]

Gao et al. formulated a macrophage–liposome conjugate delivery system to augment recruitment of macrophages for the targeted treatment of anti-atherosclerosis, through the utilisation of synergistic plaque lysis and enhanced anti-inflammatory effects. They used endogenous macrophages as drug-transporting carrier cells through membrane modification with a β-cyclodextrin (β-CD) derivative.

Chiefly through host–guest interactions between β-CD and ADA, adamantane (ADA) modified quercetin (QT)-loaded liposome (QT-NP) can be coupled with CD-MP, to develop a macrophage–liposome conjugate (MP-QT-NP). As a response action to the plaque inflammation, macrophage transports the modified liposome jointly to remarkably improve the build-up of anchored QT-NP in the aortic plaque.[128]

When simvastatin (SIM) liposomes were injected through intravenous route, there was a notable suppression in neointima formation and inhibition of the growth of monocytes/macrophages cell lines. Thin film and freeze-thaw method were utilised to synthesis mano liposomes. Cerivastatin was then encapsulated into liposomes which showcased sustained release and overall reduction in the proliferation of pulmonary artery smooth muscle cells in vitro. Additionally, the cellular cytotoxic effects were significantly less compared to the free drug. In PAH models, liposomal cerivastatin were revealed to be highly efficacious in the recovery of cardiac and lung function. Compared to the oral route, metabolic and pharmacokinetic studies implied that delivery of the cerivastatin liposomes through the inhalational route for patients with PAH could be more therapeutically superior.[129]

In a study conducted by Beretta et al, it involved the administration of both lipophilic SIM lactone and hydrophilic SIM acid via intravascular infusion route for ischemia in guinea pig brains. It was observed that SIM lactone crucially delayed the onset of ischemia and ameliorated the antioxidant capacity of the brain as compared to SIM acid. The SIM lipophilicity was a major contributor to the effect of intravascularly delivered SIM during the initial stage of cerebral ischemia.[130]

Liposomes for the targeted delivery of Atorvastatin and Curcumin to dysfunctional endothelial cells were developed by Li et al. It prompted the synergistic suppression of adhesion molecules (E-selectin and ICAM-1) and plasma lipid levels. Additionally, it also minimised the formation of foam cell and by blocking monocyte migration into the intima, it diminished the secretion of inflammatory factors (IL-6 and MCP-1). Moreover, Curcumin successfully curbed Atorvastatin associated cytotoxicity.[131]

Darwitan et al. formulated a liposomal system composed of fluocinolone acetonide with high loading capacity and sustained release. The product exhibited characteristic potent anti- inflammatory and cholesterol efflux capability in vitro. Furthermore, they also substantiated enhanced accumulation in the atherosclerotic plaques and antiatherogenic effect was observed in the Apoe mice. This FA-liposomal formulation is regarded as a novel potent atherosclerosis nano therapy for specific targeting of atherosclerotic inflammation.[132]

A platelet-mimetic hybrid liposome (P-Lipo) was developed by Song et al, through the fusion of natural platelet membrane with artificial liposomes, mainly to promote the targeted therapy of atherosclerosis. The atheroprotective drug rapamycin was used as the model drug. The hybrid P-Lipo mimicked the membranes composition of platelets and was conferred the multivalent targeting functionality with simultaneous less utilization of membrane. P-Lipo demonstrated exceptional physicochemical properties, high drug loading capacity, prolonged drug release profile and good tolerability. As a targeted delivery platform, P-Lipo featured preferential accumulation and deeper penetration into atherosclerotic plaques, which contributed to enhanced therapeutic effect of rapamycin

in an atherosclerosis model of mice.[133]

Some other liposomal formulations aimed at atherosclerotic treatment are mentioned in the table below.

Table 1: Previously made Liposomal Anti-Atherosclerotic Formulations

Formulation	Description	Reference
Doxil (Doxorubicin Liposome Injection)	This formulation has been studied for its potential anti-inflammatory effects in atherosclerosis	[134]
Atorvastatin-Loaded Liposomes	Enhance the delivery statin	[135]
Curcumin-Loaded Liposomes	The use of liposomal curcumin to reduce inflammation associated with atherosclerosis	[136]
Resveratrol-Encapsulated Liposomes	Delivering resveratrol focused on its antioxidant and anti-inflammatory properties	[137]
Nucleic Acid-Loaded Liposomes	Formulations targeting siRNA or miRNA for gene silencing related to lipid metabolism	[138]
Lipid Nanoparticles for Antisense Oligonucleotides	Investigated lipid formulations that could target genes involved in atherosclerosis	[139]

MARKETED FORMULATIONS OF LIPOSOMES

Due to their multitude of advantages, a number of drugs are increasingly being incorporated into this drug delivery system. Some of the current marketed formulations for various drugs are listed below.

Table 2: Examples of Marketed Formulations of liposomes.

Product Name	Manufacturer	Active Pharmaceutical Ingredient	References
AmBisome	(Gilead Sciences Inc.)	Amphotericin B	[140]
Onivyde	(Ipsen Biopharmaceuticals Ltd)	Irinotecan	[141]
Lyso- Thermosensitive Liposomal Doxorubicin (LTLD)	Mepac	Doxorubicin	[142]
Abelcet	(Leadient Biosciences)	Amphotericin-B Lipid Complex	[143]
Visudyne	(Bausch + Lomb)	Verteporfin	[144]
DepoDur	(Pacira Pharmaceuticals)	Extended-release epidural morphine	[145]
Exparel	(Pacira Pharmaceuticals)	Bupivacaine	[146]

CONCLUSION

Liposomal drug delivery systems represent a sophisticated, effective and highly adaptable technology for the diagnosis and treatment of atherosclerosis. Their versatility in encapsulating both hydrophilic and hydrophobic drugs offer a distinct superiority compared to other formulations. With the added benefits of enhanced bioavailability, targeted drug delivery and improved biopharmaceutical characteristics, they are rapidly growing as a means to deliver several classes of drugs in the pharmaceutical industry. The conventional dosage forms used to deliver drugs involved in the therapy of atherosclerosis present with a number of complications, most of which can be overcome by converting to liposomal formulations. A number of formulations have been developed clinically to treat atherosclerosis, mostly consisting of anti-inflammatory drugs and cholesterol-lowering agents which target the key pathological pathways in the disease. Furthermore, liposomes can be engineered to release drugs in response to specific stimuli within the atherosclerotic plaque microenvironment, optimizing therapeutic outcomes. Despite these promising developments, a host of challenges need to be fully recognized and resolved before liposomal formulations can become a key therapeutic option for atherosclerosis. The shortcomings of liposomes mainly reside in their stability issues, high clearance rate by the RES, the logistics of large scale production and the exorbitant manufacturing costs associated with their synthesis. Additionally, properly establishing their safety and toxicity data in the human body and validating targeting pathways is essential for their success. A variety of future advancements can be carried out, chiefly the development of ligand- targeted and stimuli-responsive liposomes. The field of personalized medicine can also be integrated to provide

holistic treatment to the patient and enhance the overall efficacy of treatment. Hence, it is necessary to engage in continual research, formulation and development and perform clinical trials that may contribute largely to facilitating the efficacious treatment of one of the most widely prevalent cardiovascular disease in the world.

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