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Review Article

An Overview: Methods for Preparation and Characterization of Liposomes as Drug Delivery Systems

Olga Popovska[1*], Jana Simonovska[1], Zoran Kavrakovski[2], Vesna Rafajlovska[1]

[1] Ss. Cyril and Methodius University in Skopje, Faculty of Technology and Metallurgy, Rudjer Boskovic 16, 1000 Skopje, Republic of Macedonia.
[2] Ss. Cyril and Methodius University in Skopje, Faculty of Pharmacy, Vodnjanska 17, 1000 Skopje, Republic of Macedonia.

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Abstract

Liposomes as artificially prepared vesicles have become important tools for improving delivery of a large number of drugs: antimicrobial agents, drugs against cancer, antifungal drugs, peptide hormones, enzymes, vaccines and genetic materials. Due to the differences in preparation methods and lipid compositions, liposomes can be classified according to their lamellarity, size, charge and application. The flexibility of their behavior can be exploited for the drug delivery through various routes of administration irrespective of their solubility properties. Encapsulation of drugs in liposomes has provided an opportunity to enhance the therapeutic indices of many drugs mainly through alteration in their biodistribution, targeting the drug to particular tissues. The role of liposomes as drug delivery system is to deliver drug in the controlled manner, reducing undesirable side effects improving its *in vitro* and *in vivo* activity, as well as reducing the toxicity of the drug and enhancing the efficacy of the encapsulated drug. This article provides an overview of methods for preparation of liposomes, as well as analytical methods for control physical, chemical and biological parameters for different types of drugs.

1. INTRODUCTION

An ideal delivery system delivers drug at a specified rate in the body in a certain period of time for an effective treatment[1]. Drugs used in the treatment of various diseases like cancer[2-5] and fungal diseases[6-8] can be highly toxic to normal tissues. The toxicity of these drugs may be minimized by decreasing delivery to healthy cells and tissues.

Liposomes have been considered to be excellent models of cell membranes. They show effective drug delivery which are commonly used in dermal applications[8]. Liposomes are microscopic spherical vesicles composed of one or more lipid bilayers with an aqueous core. They are formed when the lipids are dispersed in an aqueous medium by stirring, in turn giving rise to population vesicles which may reach a size range[2]. The major structural components of liposomes are phospholipids and cholesterol. The lipid bilayer is composed of phospholipids which have a hydrophilic head group and a hydrophobic tail group. The head is attracted to water, and the tail, which is made of a long hydrocarbon chain, is repelled by water[9]. Phospholipids as the main component of the liposomes can easily get integrated with the skin lipids improving drug penetration and localization in the skin layers[4]. The cholesterol incorporated in the lipid membrane helps in the increasing the stability of liposomes, as well as it reduces the permeability of the membranes. The properties of cholesterol lead to decrease the fluidity or increase the microviscosity of the bilayer[2]. Mainly, for modification of the net surface charge of the liposomal formulation charge inducers are used. The most common charge inducers are: stearylamine as cationic charge inducer[3], as well as diacetylphosphate, phosphatidyl glycerol and phosphatidyl serine as negatively charged lipids. Surfactants such as Triton X-100; Span80 are either used to lyse the liposome structure and release the entrapped drug or to reduce the surface tension[8,10-12]. Once the liposomes have reached both the aqueous and lipid phases, they can encapsulate drugs with widely varying lipophilicities. As bilayer structures, liposomes in aqueous solution are able to encapsulate hydrophilic substances in the aqueous compartment while hydrophobic substances can be accommodated in the lipid phase. Liposomes as drug delivery systems are used due to their unique characteristics like biodegradability and non-toxicity, flexibility, delivering both hydrophilic and lipophilic drugs, increasing efficacy and therapeutic index, increasing stability of entrapped drug via encapsulation protecting the entrapped drug against enzymatic degradation whilst in circulation and reducing side effects of drugs[13-16].

The liposomes containing drugs can be administrated by many routes i.e. intravenous, oral, nasal, intramuscular, pulmonary, topical, and ocular[8,9]. They can be delivered in many vesicles like creams, ointments, capsules, solutions, sprays, etc. Liposomes can be used for the treatment of many diseases: cancer[2-5,17], bacterial[18-20], fungal[6-8], ocular[21-23], vaccines[24,25], fibrinolysis[26], endocrine[27], arthritis[15], asthma[9], diabetes[16], diseases of immune system[1,10,13,28], herpes[29], drugs used to achieve relief from pain[11,30] and topical anesthesia[31].

Liposomes are classified on the basis of structural parameters, method of preparation, composition and application in biology, biochemistry, cosmetics and medicine[9]. Phospholipids which are capable of forming liposomes include both natural and synthetic phospholipids. The main sources of natural phospholipids are yolk egg and soya bean although they can be obtained from plant oils such as olive oil[32]. Liposomes can be classified as conventional, pH sensitive liposomes, cationic liposomes, immunoliposomes, temperature or heat sensitive liposomes, magnetic liposomes and sterically stabilized "stealth" liposomes[14, 33]. The most common used phospholipids in preparation of liposomes are: phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI),

Corresponding Author:
Olga Popovska,
Ss. Cyril and Methodius University in Skopje,
Faculty of Technology and Metallurgy
Rudjer Boskovic 16, 1000 Skopje, Republic of Macedonia
Email: olga_popovska@yahoo.com

dipalmitoylphosphatidylcholine (DPPC), 1, 2-dioleoyl-*sn*-glycero-3-phospho-L-serine, distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylserine, dipalmitoylphosphatidylglycerol dioleoylphosphatidylethanolamine (DOPE).

The conventional liposomes are typically composed of phosphatidylcholine and cholesterol. On the other hand, "stealth" liposomes are lipid bilayers coated with polyethylene glycol (PEG)[34]. The "stealth" liposomes provide better stabilization and additional protection of the entrapped substances. They allow longer circulation time for the drug delivery mechanism, also[14,35]. The importance of a prolonged period of time can be achieved by sustained release of the drug[2,7]. In the literature the use of PEG is avoided due to unknown effects in a long term of treatment[36]. A low-molecular weight PEG is not toxic, but it is not biodegradable. Various alternatives to PEG have been developed such as amino acids: glycine, β-alanine and γ-aminobutyric acid[36,37].

Generally, based on size the liposomes are classified as: small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and multilamellar vesicles (MLV)[38]. The SUV are usually smaller than 50 nm, while LUV are larger than 50 nm. Multilamellar vesicles (MLV) consisted of more bilayers ranged in size from 500 nm to 10000 nm. The unilamellar liposomes have a single phospholipid bilayer sphere enclosing aqueous solution, while the multilamellar vesicles have onion structure which form one inside the other, creating a multilamellar structure of concentric phospholipid spheres separated by layers of water[5,26].

There are two types of targeting the liposomes: passive and active[5]. Passive targeting exploits the natural tendency of some cells i.e. Kupffer cells in the liver and the macrophages of the mononuclear phagocyte system (MPS) to phagocyte foreign microparticles such as liposomes[5]. The effect of this phagocytosis is the passive incorporation of the entrapped drug into a desirable organ[5]. The active targeting requires the formation of immunoliposomes prior to their specific interaction in the target tissues[5]. Although the presence of negatively charged lipids in liposomes including phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidylglycerol (PG) results in rapid uptake by mononuclear phagocyte system (MPS), the relationship between the presence of charged lipids and circulation lifetimes is extremely complex[14,39]. The longer a liposome can circulate by avoiding uptake in the liver, the greater the possibility that it leaves the vascular system at the sites where the blood vessels are porous[1,23]. The encapsulated drugs can be released by different mechanisms such as: fusion, difference in pH and temperature[2]. The rate of release from liposomal formulations is drug dependent. The therapeutic benefits will be achieved if the drug is retained in the liposomes for several hours after administration[40].

The main instabilities of liposomes are related to their possibility of hydrolysis, as well as peroxidation reactions and ability of aggregation[6]. Chemical instability of liposomes may be caused by hydrolysis of ester bond and/or peroxidation of unsatured acyl chains of lipids via free radical reactions. Peroxidation reactions can be avoided by selecting lipids with only saturated bonds, storage under inner environment, as well as addition of antioxidants such as α-tocopherol, quercetin and coenzyme Q (CoQ) and chelating agents[6,41,42]. The literature date show that by using grape seed extracts in preparation of chitosan-liposomes the chemical stability against lipid oxidation is improved[43]. The kinetic hydrolysis of phospholipids depends on temperature, pH and bilayer rigidity[28,29,44,45]. Liposome formulations can be improved in their stabilization by optimizing the size distribution, pH and ionic strength[9]. It is found[45] that an optimal stability for phosphatidylcholine liposomes is 6.5. Lyophilization is preferred method for stabilization of liposomes in their storage avoiding fusion and leakage of encapsulated drug[23]. This method is employed to remove water from products in the frozen state at extremely low pressures[44]. The process is used to dry products that are thermolabile and could be destroyed by heat-drying[44,46]. Various types of sugars like disaccharide trehalose are involved as cryoprotectants with role to maintain liposomes distribution of particle size and to protect liposomes against aggregation[28,46,47].

The ability of liposomes to retain the drug is assayed[4,6,22] by keeping the liposomal formulations at different temperature conditions: 4 ºC, 8 ºC; 25 ºC and 45 ºC during 4 to 5 weeks[4-6] and for a period of two to three months[1,8,14,19,21,22,29]. The studies of liposomal stability indicated that at lower temperature the drug liposomal formulation show better stability than at higher temperatures[8,22,29].

Sterilization of liposomes is very important to improve their safety for clinical use[48]. The main problems in the sterilization process are in line with the high thermolability of liposomes, as well as, they are sensitive to heat, radiation and/or chemical sterilization agents[8,48]. The most common used methods for sterilization are: autoclaving without substantial degradation of phospholipids and γ-irradiation[21,48].

The choice of an appropriate liposome type depends on physicochemical properties of the substance to be entrapped, its effective concentration, potential toxicity and internal volume[18]. The encapsulation efficiency greatly depends on liposomal content, lipid concentration, method of preparation and the drug which is used. Different methods for preparation of liposomes lead to formation of liposomes with different sizes and characteristics.

2. METHODS FOR PREPARATION OF LIPOSOMES

The main goal of an ideal method of liposome formulation is to obtain efficient drug entrapment, narrow particles size distribution and long term stability of liposome products. The general procedure for all methods of liposomes preparation involves hydrating of the lipid, followed by sizing of the particles and removing of the non encapsulated drug. There are two types used for the preparation of liposomes: passive loading mechanical dispersion methods and active loading methods. The most common used methods in the preparation for liposomes are: thin-film hydration method, microemulsification, sonication, membrane extrusion, freeze-thawed method, ether injection method, ethanol injection method, reverse phase evaporation method, dehydration-rehydration, and calcium-induced fusion method [9,44,46,48]. In the passive loading method the drug is encapsulated by introducing an aqueous phase of a water-soluble drug or an organic phase of a lipid-soluble drug before or at some stage during the preparation of the liposomes. The high drug encapsulation efficiency can be achieved by using passive loading method for lipid-soluble drugs with a high affinity to the lipid membrane. In the active loading method, the drugs can be loaded by creating diffusion gradients for the ions or drugs across the external and internal aqueous phases. The classification of liposomes based on methods for their preparation depends on using the organic solvent, obtaining liposomes with different lamellarity, transforming the size and applications of the liposomes (Table 1). The phospholipids play important role in the preparation of liposomes, as well as in their stability[48]. In table 2 are presented most commonly used phospholipids.

Table 1: Methods for preparation of drug delivery liposomes

Drugs liposome formulation	Method	Type of liposome*	Reference
Drugs against cancer			
Doxorubicin	Lipid-film hydration method and extrusion	MLV	2
Bis-demethoxy Curcumin analogue	Thin-film hydration method and sonication	MLV SUV	3
Tamoxifen	Thin-film hydration method	MLV	4
5-fluorouracil	Lipid-film hydration method, extrusion, ethanol injection and reverse phase evaporation	MLV MLV, LUV, SUV MLV, LUV	5
Vinblastine sulphate	Thin-film hydration method and sonication	MLV SUV	28
Antifungal drugs			
Fluconazole	Thin-film hydration method	MLV	6
Amphotericin B	Thin-film hydration method	MLV	7
Clotrimazole	Rotary evaporation method	MLV	8
Antibiotic drugs			
Mafenide acetate	Solvent evaporation and microencapsulation	MLV SUV	19
Amikacin	Reverse phase evaporation method	MLV, LUV	20
Immunosuppressive drugs			
Tacrolimus (Fk-506)	Thin-film hydration method	MLV	1
Sirolimus	Thin-film hydration method	MLV	13
Ophthalmic drugs			
Acetazolamide	Reverse phase evaporation and thin-film hydration method	MLV, LUV MLV	21
Brimonidine tartrate	Thin-film hydration method and sonication	MLV SUV	22
Analgesic drugs			
Nimesulide	Ethanol injection method and rotary evaporation method	MLV, LUV, SUV MLV	30
Ketorolac tromethamine	Thin-film hydration method	MLV	11
Antifibrinolytic drugs			
Tranexamic acid	Chloroform film and sonication	SUV	26
Vaccines			
Tetanus toxoid diphtheria toxoid	Reverse phase evaporation method	MLV, LUV	25
Hormone drugs			
Cyproterone acetate	Thin-film hydration method	MLV	27
Potential drugs as oral insulin			
Sodium glycocholate Metformin hydrochloride	Reverse phase evaporation Thin-film hydration method	MLV, LUV MLV	16 18

*SUV-small unilamellar vesicles; *LUV-large unilamellar vesicles; *MLV-multilamellar vesicles

Table 2: Phospholipids used in formulations of drug delivery liposomes

Drugs liposome formulation	Type of phospholipids	Reference
Tacrolimus (Fk-506)	Soya phosphatidylcholine	1
Doxorubicin	DPPC*, DSPC*, PC*	2
1,7- *bis*(2-hydroxyphenyl)-1,6-heptadiene-3,5-dione	Soya phosphatidylcholine	3
Tamoxifen	Soya phosphatidylcholine	4
5-fluorouracil	DPPC*	5
Fluconazole	Soya phosphatidylcholine	6
Amphotericin B	Soya phosphatidylcholine	7,14
Clotrimazole	Soya phosphatidylcholine	8
Salbutamol	PC*	9
Nevirapine	PC* (egg)	10
Ketorolac tromethamine	Epikuron 200 (95 % PC*)	11
Cisplatin	PE* (from sheep brain)	12
Sirolimus	DPPC*, DOPE*	13
Sodium glycocholate	Soya phosphatidylcholine	16
Metformin hydrochloride	PC*	18
Mafenide acetate	PC*	19
Amikacin	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine	20
Acetazolamide	PC* (dried egg yolk)	21
Brimonidine tartrate	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine	22
Pilocarpine nitrate	PC*	23
Tetanus toxoid and diphtheria toxoid	Soya phosphatidylcholine	25
Tranexamic acid	Soya phosphatidylcholine	26
Cyproterone acetate	PC* (egg)	27
Vinblastine sulphate	Hydrogenated soya phosphatidylcholine, Dipalmitoyl glycero-phosphocholine Dipalmitoyl glycero-phosphatidic acid	28
Acyclovir	PC*	29
Nimesulide	DPPC*	30
Clindamycin and tretinoin	Soya phosphatidylcholine	41
Sodium diclofenac	Soya phosphatidylcholine	49
Pentoxifylline	PC*	50

*DPPC-dipalmitoylphosphatidylcholine; *DSPC-distearoylphosphatidylcholine; *PC-phosphatidylcholine; *DOPE-dioleoylphosphatidylethanolamine; *PE-phosphatidylethanolamine

2.1 Thin-Film Hydration Method

The thin-film hydration procedure is the most common and simple method for preparation of MLV by dissolving the phospholipids in the organic solvents: dichloromethane[6], chloroform[3,7,14], ethanol[2] and chloroform-methanol mixture (2:1 v/v; 9:1 v/v; 3:1 v/v)[4,5,13].

A thin and homogeneous lipid film is formed[3-6] when solvent is evaporated under vacuum at the temperature: 45-60 ºC. Nitrogen gas is involved in order to completely remove the residual solvent[2,49]. A solution of distilled water[5,12], phosphate buffer[18], phosphate saline buffer[7,13] at pH 7.4 and normal saline buffer[2-4] are used in hydration step. The time for the hydration process[2-4] varied from 1 h to 2 h at the temperature 60-70 ºC. In order to obtain full lipid hydration, the liposomal suspension is left overnight at 4 ºC[6,24].

The thin-film hydration method can be used for all different kinds of lipid mixtures. The main drawbacks of the method are related to low encapsulation, difficulty of scaling up and the size distribution is heterogeneous[6,48].

2.2 Injection Methods

2.2.1 Ether Injection Method

In ether injection method a solution of lipids is dissolved in ether or diethyl ether/methanol mixture which is slowly injected to an aqueous solution of the material to be capsulated. The subsequent removal of the organic solvent under reduced pressure leads to the formation of liposomes[30,48].

The main disadvantage of the method is heterogeneous population and the exposure of compounds to be encapsulated to organic solvents or high temperature[9].

2.2.2 Ethanol Injection Method

In ethanol injection method the ethanolic lipid solution is rapidly injected to a vast excess of preheated distilled water[30] or TRIS-HCl buffer[5]. The incorporation of the drug in liposomal vesicle depends on its hydrophilic/hydrophobic character. Nimesulide as lipid soluble component incorporates better in liposomes than 5-fluorouracil which migrates to external aqueous phase[5,30]. The main advantage of ethanol injection method is including of non harmful solvent as ethanol, as well as easy scale up of the method. The possibility of formation of azeotrope with water reduces its applicability[30,48].

2.3 Sonication Method

The sonication method is based on size transformation and involves the subsequent sonication of MLVs prepared by thin-film hydration method, using sonic energy usually under an inert atmosphere including nitrogen or argon. The sonication method enables homogenous dispersion of small vesicles using bath type or probe type sonicator with a potential for greater tissue penetration. The probe tip sonicator delivers high energy to the lipid suspension. The possibility of overheating of the lipid suspension causes degradation[12,22,49]. Sonication tips tend to release titanium particles into the lipid suspension which must be removed by centrifugation prior to use. The bath sonicators are the most widely used instrumentation for preparation of SUV[5,14,19,21,23,24]. They are used for large volume of dilute lipids. The oxidation of unsaturated bonds in the fatty acid chains of phospholipids and hydrolysis to lysophospholipids and free fatty acids, as well as denaturation of thermolabile substances and very low encapsulation efficiency of internal volume are the main drawbacks of the method[14,48].

2.4 High-Pressure Extrusion Method

MLVs prepared by thin-film hydration method are repeatedly passed through filters polycarbonate membranes reducing the liposome size in high-pressure extrusion method[9,10]. The liposomes are prepared using thin-film hydration method subsequently using an extruder for ten cycles to obtain extruded liposomes with uniform diameters[10].

2.5 Reverse-Phase Evaporation Method

The reverse-phase evaporation method is used with the organic solvents such as diethyl ether/isopropyl ether or mixture of diethyl ether and chloroform (1:1 v/v)[5] and a mixture of chloroform-methanol (2:1 v/v)[21] containing phospholipids. The organic phase should be immiscible with aqueous phase, thus an oil/water emulsion is created. Phosphate buffer saline[21,25] or citric-Na_2HPO_4 buffer[3] is added to aqueous phase with aim to improve the efficiency of liposome formulations[5]. The formation of liposomes is allowed by continued rotary evaporation of the organic solvents under vacuum[5,16,21,25]. The main advantage of the method is a very high encapsulation rate[20,21]. The main drawback of the method is the possibility of remaining the solvent in the formulation and it has difficulties to scale up[5,21].

2.6 Calcium-Induced Fusion Method

The calcium-induced method is based on adding of calcium to SUV. The formation of multilamellar vesicles is as result of fusion. The addition of ethylenediaminetetraacetic acid (EDTA) to the preparations results in the formation of LUV liposomes[15]. The preparation of LUV liposomes can be obtained only from acidic phospholipids[9,15].

2.7 Dehydration-Rehydration Method

The method of dehydration-rehydration is used as method for the preparation of liposomes, also[44,51]. The small unilamellar vesicles which are composed of phosphatidylcholine, 1,2-dioleoyl-3-(trimethylammonium) propane, cholesterol and plasmid DNA are prepared by sonication method[51]. The obtained formulation is frozen and left freeze-dried overnight. The formation of multilamellar dehydration-rehydration vesicles containing DNA in their structure due to the bound of the cationic charges of the inner bilayers is as a result of a controlled rehydration of the dry powders[51].

2.8 Freeze-Thaw Method

The method of freezing and thawing is introduced for increasing the trapped volume of liposomal preparations. The freeze-thaw method is dependent on the ionic strength of the medium and the phospholipid concentration[35]. It influences to a physical disruption of lamellar structure leading to formation of unilamellar vesicles. The unilamellar vesicles are rapidly frozen followed by slow thawing[35,52], while the freeze and thawing cycles are repeated[53,48]. The preparation of MLV propranolol liposomes by freeze-thaw method is described in the literature[53]. The liposomal propranolol formulation is prepared by using distearoylphosphatidylcholine and dimyristoylphosphatidylcholine as phospholipids in phosphate buffered saline buffer, followed by six freeze-thaw cycles[53].

2.9 Microfluidization

A method based on microfluidization i.e. microemulsification is used for the large scale manufacture of liposomes. The preparation of antibiotic liposomes by thin-layer hydration method followed by sonication with a bath-type sonicator and microfluidization in order to achieve partial homogenization was described by Boltič *et al*.,[54]. The process of microfluidization is reproducible and yield liposomes with good aqueous phase encapsulation [35,55].

2.10 Supercritical Fluids (SCF) in the Preparation of Liposomes

Supercritical fluids are introduced in the preparation of liposomes to overcome existing problems with conventional methods such as requiring a high amount of toxic organic solvents and limited laboratory scale production[56-59]. The most common used supercritical fluid in the preparation of liposomes in pharmaceutical field is supercritical carbon dioxide[59]. It has several advantages: non-toxicity, non-flammability, recyclable and easy removal from the solvent, operation at moderate temperatures and avoiding degradation of the product in an inert atmosphere[58,60]. The use of SCF allows controlling of extraction condition by variation of temperature, pressure or adding modifier solvents as cosolvents: acetone, ethanol, methanol, dichloromethane and ethyl acetate[60]. A comparison between thin-film hydration method and SCF method is reported by Karn *et al*.,[58]. A mixture of phosphatidylcholine, cholesterol and cyclosporin A is dissolved in ethanol followed by pumping supercritical carbon dioxide to the reaction vesicle in SCF method. Distilled water in hydration step in thin-film hydration method is used[58].

3. ANALYTICAL METHODS FOR CONTORL OF LIPOSOMAL FORMULATIONS

Physico-chemical and biological characteristics of the liposomal formulations, as well as their stability and drug entrapment efficiency are controlled by different analytical methods[6,9]. The most important parameters of liposome include size distribution, lamellarity, surface charge, drug entrapment efficiency,

phospholipid concentration, cholesterol concentration, osmolarity, sterility and pyrogenicity[9].

3.1 Physical Parameters

The control of physical parameters are based on measuring vesicle shape, surface morphology, mean vesicle size and size distribution, surface charge, electrical surface potential and surface pH, lamellarity, phase behavior, percent of free drug/percent capture and drug release[19,38]. The visual appearance of the liposome suspension can be ranged from translucent to milky, depending on the composition and particle size[44]. The determination of liposomal size distribution is usually measured by dynamic light scattering while the lamellarity of liposomes is measured by electron microscopy or by spectroscopic method (Table 3). Physical stable liposomal formulations preserve liposome size distribution. Physical instability may be caused by drug leakage from the vesicles and/or aggregation or fusion of vesicles to form larger particles[44].

Table 3: Physical characterization of drug delivery liposomes

Drugs liposome formulation	Parameter	Method	Reference
Drug against cancer			
Doxorubicin	Morphology and size determination	Transmission electron microscopy	2
Bis-demethoxy Curcumin analogue	Size distribution and zeta potential Entrapment efficiency	Photon correlation spectroscopy Spectrophotometry	3
Tamoxifen	Microscopy and size distribution profile	Optical microscopy Light scattering based on laser diffraction method	4
Cisplatin	Microstructure Mean vesicle size and zeta potential	Transmission electron microscopy Laser scattering method	12
Antifungal drugs			
Fluconazole	Shape and lamellarity Particle size and particle distribution	Microscope Laser diffraction method	6
Amphotericin B	Morphology	Transmission electron microscopy	7
Analgesic drugs			
Ketorolac tromethamine	Average diameter, size distribution and zeta potential Surface morphology	Photon correlation spectroscopy Transmission electron microscopy	11
Nimesulide	Mean particle size analysis	Photon correlation spectroscopy	30
Ophthalmic drugs			
Acetazolamide	Particle size and size distribution	Laser diffraction method	21
Antifibrinolytic drugs			
Tranexamic acid	Particle size and size distribution	Laser scattering method	26
Antibiotic drugs			
Amikacin	Size and homogeneity	Negative-stain electron microscopy	20
Vaccines			
Tetanus toxoid and diphtheria toxoid	Morphology and size distribution	Optical microscope and Transmission electron microscopy	25
Drugs against asthma			
Salbutamol	Morphology and particle size	Optical microscope	9

3.1.1 Drug Entrapment Efficiency

The determination of drug entrapment efficiency is of great importance because of concluding the justification of introducing the liposomes[13,61]. The extraction of a liposome-encapsulated pellet is done by dispersing in organic solvent: chloroform[3], chloroform-methanol (2:1 v/v)[4], methanol[6,28], ethanol[21] or in phosphate buffer saline[22] and phosphate buffer[50] subsequently analyzing drug content. The free drug can be analyzed in the supernant using appropriate analytical method: spectrophotometry[8,10,21,22,26,29,30,50] or chromatographic methods[14,16,20,22,41,62]. The gel permeation chromatography is used, also[5,11]. Ultra-centrifugation is introduced in order to separate the entrapped drug from the free drug[1,3,27,28].

*3.1.2 High P*erformance *Liquid Chromatography (HPLC)*

The HPLC method has been widely applied to the determination of drugs in liposome formulation. Some of these methods involve the analysis of encapsulated drug in liposome[62] or drugs encapsulated in liposomes in biological samples such as plasma[3] and liver[3]. Solid-phase extraction (SPE) is of great interest in the separation of liposomal and non-liposomal drug forms. Separation is based on the property of liposomes to cross reversed-phase C_{18} silica gel cartridges without being retained, while a non-liposomal drug is retained on the stationary phase[63] which are subsequently determined by high-pressure liquid chromatography (HPLC) or electrophoresis[25].

The detection modes extensively used in HPLC of drug delivery liposomes are: UV[62], fluorimetric[20,63] and mass spectrometry (MS)[1]. The HPLC methods for control of liposomal formulations are based on adjusting parameters such as mobile phase and flow speed (Table 4).

3.1.3 Size Exclusion Chromatography (SEC)

The size exclusion chromatography has been used for fractionation of liposomes[12]. This method is applied to the determination of cisplatin[12] (Hwang et al., 2007), ceftazidime[64] and cefepime[64] encapsulated in liposomes. The unentrapped cisplatin is removed on a Sephadex G-15 column using distilled water[12], while for the unentrapped quantity of ceftazidime and cefepime a Sephadex G-50 column is used[64].

3.1.4 High-Performance Thin-Layer Chromatography (HPTLC)

A HPTLC method for the determination of ketorolac tromethamine is introduced by Nava *et al*.,[11]. The content of the drug is determined by spotting samples on TLC plates, Alugram® Sil G/UV_{254} and developing in ethyl acetate:chloroform:acetic acid (8:3:0.1).

3.1.5 Capillary Electrophoresis (CE)

The capillary electrophoresis as an effective tool is employed to investigate the properties of liposomes such as membrane fluidity and rigidity, phospholipid distribution in the membrane, membrane disruption, size distribution and surface charge density[65,66]. The electromigration of liposomes is a complex process and several aspects such as liposome radius, the thickness of the electric double layer and the surface charge are important variables that affect liposome separation[66]. A capillary electrophoresis-inductively coupled plasma mass spectrometry (CE-ICP-MS) is used for the separation of the free oxaliplatin drug substance from liposome entrapped oxaliplatin[67]. The ICP-MS is employed to monitor $^{31}P^{+}$, $^{195}Pt^{+}$, $^{127}I^{+}$, and $^{115}In^{+}$ signals in order to separate the free unencapsulated oxaliplatin. Hautala *et al*., studied the effect of calcium on phospholipid coatings in fused silica capillaries[68].

Table 4: Conditions for HPLC analysis for efficiency of drug delivery liposomes

Drugs liposome formulation	Detector type	Chromatographic column	Mobile phase	Reference
Tacrolimus (Fk-506)	HPLC-MS	Phenominex (Gemini) - ODS-(150 mm x 4.6 mm, 5µm particle size)	Acetonitrile-water (80:20 v/v)	1
Bis-demethoxy curcumin analogue	UV	C-18 ODS (250 mm x 4.6 mm, 5µm particle size)	Methanol-water (80:20 v/v/)	3
Sirolimus	UV	KNAUER column C-18 (150 x 4.6 mm, 5µm particle size)	Acetonitrile and ammonium acetate buffer (70:30 v/v)	13
Amphotericin B	UV	C-18, Supelco, (250 mm x 4.6 mm, 5 µm particle size)	Acetonitrile and EDTA (40:60 v/v)	14
Sodium glycocholate	UV	C-18 (Zorbax® 150 mm x 4.6 mm, 5 µm particle size)	Acetonitrile-H_3PO_4 (26:74 v/v)	16
Amikacin	Fluorimetric detection	Reverse phase Kromasil 100 C-18 columns (15 x 0.46 cm)	Methanol-water (69:31 v/v/)	20
Clindamycin	UV	LiChrospher 100 RP-18 (125 mm x 4 mm, 5 µm particle size)	Phosphate buffered saline-acetonitrile (55:45 v/v)	41
Tretinoin	UV	LiChrospher 100 RP-18 (125 mm x 4 mm, 5 µm particle size)	Water-glacial acetic acid-acetonitrile (15:0.5:84.5 v/v/v)	41
Amphotericin B	UV	C-18 µBondapac column (300 mm x 4.6 mm, 5 µm particle size)	Acetonitrile and ethylenediaminetetraacetic acid disodium salt (45:55 v/v)	62
Doxorubicin	Fluorimetric detection	Diamonsil C-18 column (200 mm x 4.6 mm, 5µm particle size)	Methanol-water (62:38 v/v)	63

3.1.6 In Vitro Drug Release Study

In vitro release studies are usually based on dialysis method[2]. These studies are developed in validated diffusion cell across either cellophane[18,22,23,29,30] or dialysis membrane[2,3,8,10,21]. An aliquot of prepared formulation is placed in a dialysis membrane at the temperature of 37 ºC and the sample is assayed for drug realizing by UV spectrophotometer[6,7,69]. The receptor medium consists of acetate buffer[6] at pH 5, citrate-phosphate buffer[12], phosphate buffer[7] at pH 7.4 and the donor medium consists of liposomal formulation. Drug released in the case of using dialysis membrane is monitored[3,8,21] for 30 min or 8 hours. The diffusion studies are carried by using Franz diffusion cell, also[1,4,6,7,12,27,29].

3.1.7 Zeta Potential (ζ-Potential)

The characterization of the surface charges property of microspheres is determined by measuring zeta potential[30]. The higher value of zeta potential implies a greater colloidal stability and results in inhibiting the aggregation of liposomal formulation[24]. It is reported that microspheres with a zeta potential above (+/-) 30 mV show stabilization in suspension[3,30,70]. Zetasizer is used for measuring the zeta potential of the liposomes[3,28,30] by applying voltage across a pair of electrodes at either end of a cell containing the particle dispersion. Charged particles are attracted to the oppositely charged electrode. The particles move in an electrical of known strength in the interference pattern of two laser beams and produce scattered light which depends on the speed of the particles[12,24].

3.1.8 Differential Scanning Calorimetry (DSC) Study

In this method drug loaded multilamellar liposomes are submitted to DSC analyzer. This method is used to determine phase transition temperature of phospholipids sample. The temperature of maximal excess heat capacity is defined as the phase transition temperature[6,10]. Thermograms are obtained[6,21,49,50] at a scanning rate of 10 ºC/min or 20 ºC/min. Each sample is scanned[49,50] between 20 ºC to 200 ºC or 25 ºC to 500 ºC.

3.2 Chemical Parameters

Chemical analysis of liposomes is important to determine the purity and potency of various liposomal constituents. The most common chemical parameters which are analyzed are: phospholipid concentration, cholesterol concentration, phospholipid peroxidation, phospholipid hydrolysis, cholesterol auto-oxidation and osmolarity[2,3,15]. The quantification of phospholipids is important due to control the efficiency of the preparation method. Phospholipids content in liposomes was determined by Stewart's method based on the formation of a colored complex between phospholipids and ammonium ferrothiocyanate reagent[2,3]. The concentration of cholesterol is determined[3] by colorimetric assay of a colored complex formed in the reaction between cholesterol and freshly prepared acetic anhydride-concentric sulfuric acid mixture (20:1) at 680 nm or by dissolving cholesterol in glacial acetic acid[15]. An isocratic high-performance liquid chromatographic method (column ASTEC diol; 250 mm x 4.6 mm, 5 µm particle size) and mobile phase: chloroform–methanol–aqueous ammonium acetate (71:26:3 v/v) is applied for a simultaneous determination of cholesterol, cardiolipin, and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine in various pharmaceutical formulations is employed in the literature[71].

3.3 Biological Parameters

The importance of determining biological parameters is helpful in determining the safety of formulation for therapeutic application. Sterility, pyrogenicity and animal toxicity are determined during the biological characterization of the liposomes[7,8,12,14,21-23].

4. CONCLUSION

A delivery system that reduces the drug toxicity while increasing its therapeutic index is of great importance. The drug delivery systems such as liposomes are developed when an existing formulation of the drug is not acceptable. The new formulation offers safety over the existing formulation. The success of liposomes as drug carriers has been reflected in a number of liposome-based formulations due to delivering of higher drug concentration. Although their potential applications require specific condition for their preparation, as well as sterility, they are promising candidates in the drug delivery because of the ability of targeting to specific cells or tissues, increasing efficacy and therapeutic index and reducing the toxicity of the encapsulated drugs. It will be of great interest the choice of the method of liposome manufacture and lipid layer composition which can directly affect drug encapsulation, release, permeation, absorption, distribution, metabolism, and elimination, as well as its toxicity profile.

REFERENCES

1. Patel SS, Patel MS, et al "Development and evaluation of liposomes for topical delivery of tacrolimus (Fk-506)" *J Sci Res*, 2010, 2 (3): 585-596.
2. Achim M, Precup C, et al "Thermosensitive liposomes containing doxorubicin. Preparation and *in vitro* evaluation" *Farmacia*, 2009, 57 (6): 703-710.
3. Aukunuru J, Joginapally S, et al "Preparation, characterization and evaluation of hepatoprotective activity of an intravenous liposomal formulation of *bis*-demethoxy curcumin analogue (BDMCA)" *Int J Drug Dev & Res*, 2009, 1 (1): 37-46.
4. Bhatia A "Tamoxifen in topical liposomes: development, characterization and *in vitro* evaluation" *J Pharm Pharmaceut Sci*, 2004, 7 (2): 252-259.

5. Da Costa CAM, Moraes AM "Encapsulation of 5-fluorouracil in liposomes for topical administration" *Maringá*, 2003, 25 (1): 53-61.
6. Chanda H, Das P, et al "Development and evaluation of liposomes of fluconazole" *JPBMS*, 2011, 5 (27): 1-9.
7. Devi M, Kumar MS, et al "Amphotericin-B loaded vesicular systems for the treatment of topical fungal infection" *IJRAPR*, 2011, 4: 37-46.
8. Kumar R, Rana AC, et al "Formulation and evaluation of elastic liposomes of clortimazole" *Int J Drug Dev & Res*, 2012, 4 (3): 348-355.
9. Nidhal KM, Athmar DH "Preparation and evaluation of salbutamol liposomal suspension using chloroform film method" *Mustansiriya Medical Journal*, 2012, 11 (2): 39-44.
10. Ramana LN, Sethuraman S, et al "Development of a liposomal nanodelivery system for nevirapine" *J Biomed Sci*, 2010, 17: 1-9.
11. Nava G, Piñón E, et al "Formulation and *in vitro*, *ex vivo* and *in vivo* evaluation of elastic liposomes for transdermal delivery of ketorolac tromethamine" *Pharmaceutics*, 2011, 3: 954-970.
12. Hwang TL, Lee WR, et al "Cisplatin encapsulated in phosphatidylethanolamine liposomes enhances the *in vitro* cytotoxicity and *in vivo* intratumor drug accumulation against melanomas" *J Dermatol Sci*, 2007, 46: 11-20.
13. Ghanbarzadeh S, Valizadeh H, et al "Application of response surface methodology in development of sirolimus liposomes prepared by thin film hydration technique" *BioImpacts*, 2013, 3 (2): 75-81.
14. Jadhav MP, Nagarsenker MS, et al "Formulation and evaluation of long circulating liposomal amphotericin B: a scinti-kinetic study using ^{99m}Tc in BALB/C mice" *Indian J Pharm Sci*, 2011, 73 (1): 57-64.
15. Mulye C, Mishra R, et al "Formulation and evaluation of liposome mediated drug delivery" *UJP*, 2013, 2 (2): 156-160.
16. Niu M, Lu Y, et al "Liposomes containing glycocholate as potential oral insulin delivery systems: preparation, *in vitro* characterization, and improved protection against enzymatic degradation" *Int J Nanomedicine*, 2011, 6: 1115-1166.
17. Song J, Shi F, et al "Formulation and evaluation of celastrol-loaded liposomes" *Molecules*, 2011, 16: 7880-7892.
18. Divakar P, Kumar DP, et al "Formulation and *in vitro* evaluation of liposomes containing metformin hydrochloride" *IJRPBS*, 2013, 4 (2): 479-485.
19. Makhmalzadeh BS, Azh Z, et al "Preparation and evaluation of mafenide acetate liposomal formulation as eschar delivery system" *Int J Drug Dev & Res*, 2011, 3 (4): 129-140.
20. Mirzaee M, Owlia P, et al "Comparison of the bactericidal activity of amikacin in free and liposomal formulation against gram-negative and gram-positive bacteria" *JJNPP*, 2009, 4 (1): 1-7.
21. Hathout RM, Mansour S, et al "Liposomes as an ocular delivery system for acetazolamide: *in vitro* and *in vivo* studies" *AAPS PharmSciTech*, 2007, 8 (1): E1-E12.
22. Prabhu P, Kumar N, et al "Preparation and evaluation of liposomes of brimonidine tartrate as an ocular drug delivery system" *Int J Res Pharm Sci*, 2010, 1(4): 502-508.
23. Rathod S, Deshpande SG "Design and evaluation of liposomal formulation of pilocarpine nitrate" *Indian J Pharm Sci*, 2010, 72 (2): 155-160.
24. Channarong S, Chaicumpa W, et al "Development and evaluation of chitosan-coated liposomes for oral DNA vaccine: the improvement of Peyer's patch targeting using a polyplex-loaded liposomes" *AAPS PharmSciTech*, 2011, 12 (1): 192-200.
25. Mishra N, Gupta PN, et al "Liposomes as adjuvant for combination vaccines" *Indian J Exp Biol*, 2007, 45: 237-241.
26. Manosroi A, Podjanasoonthon K, et al "Development of novel topical tranexamic acid liposome formulations" *Int J Pharm*, 2002, 235: 61-70.
27. Mohammadi-Samani S, Montaseri H, et al "Preparation and evaluation of cyproterone acetate liposome for topical drug delivery" *Indian J Pharm Sci*, 2009, 5 (4): 199-204.
28. Patel H, Patel H, et al "Preparation and characterization of calcium phosphate coated vinblastine sulfate liposomes" *IJPRBS*, 2013, 2 (2): 263-280.
29. Manjunatha N, Naidu GP, et al "Preparation and evaluation of liposomes of an antiviral drug" *IJNDD*, 2009, 1 (1): 25-31.
30. Kumar A, Badde S, et al "Development and characterization of liposomal drug delivery system for nimesulide" *Int J Pharm Pharm Sci*, 2010, 2 (Suppl 4): 87-89.
31. Pandey RS "Formulation of benzocaine loaded PG-liposomes for enhanced skin delivery" *AJPMS*, 2012, 2 (5): 78-82.
32. Malisiova F, Hatziantoniou S, et al "Liposomal formulations from phospholipids of Greek almond oil. Properties and biological activity" *Z Naturforsch C*, 2004, 59 (5-6): 330-334.
33. Sharma S, Mishra L, et al "Liposomes: vesicular system an overview" *Int J Pharm Pharm Sci*, 2010, 2 (4): 11-17.
34. Panwar P, Pandey B, et al "Preparation, characterization, and *in vitro* release study of albendazole-encapsulated nanosize liposomes" *Int J Nanomedicine*, 2010, 5: 101-108.
35. Gaurav R, Tejal S "Liposomal drug delivery system: an overview" *IJPBA*, 2011, 2 (6): 1575-1580.
36. Riché EL, Erickson BW, et al "Novel long-circulating liposomes containing peptide library-lipid conjugates: synthesis and *in vivo* behavior" *J Drug Target*, 2004; 12 (6): 355-361.
37. Torchilin VP, Levchenko TS, et al "Amphiphilic poly-*N*-vinylpyrrolidones: synthesis, properties and liposome surface modification" *Biomaterials*, 2001, 22: 3035-3044.
38. Prazanth VV, Maharshi S, et al "Liposomes: an overview" *IJPI's Journal of Pharmaceutics and Cosmetology*, 2012, 2 (8): 67-74.
39. Drummond DC, Meyer O et al "Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors" *Pharmacol Rev*, 1999, 51 (4): 691-743.
40. Yamauchi M, Tsutsumi K, et al "Release of drugs from liposomes varies with particle size" *Biol Pharm Bull*, 2007, 30 (5): 963-966.
41. Malaekeh-Nikouei B, Golmohammadzadeh S, et al "Preparation and characterization of liposomes encapsulated with clindamycin and tretinoin" *IJCP*, 2011, 6 (4):1-4.
42. Shaji J, Iyer S "Preparation, optimization and *in-vivo* hepatoprotective evaluation of quercetin liposomes" *Int J Curr Pharm Res*, 2012, 4 (2): 24-32.
43. Gibis M, Rahn N, et al "Physical and oxidative stability of uncoated and chitosan-coated liposomes containing grape seed extract" *Pharmaceutics*, 2013, 5: 421-433.
44. Anwekar H, Patel S, et al "Liposome-as drug carriers" *Int J of Pharm & Life Sci*, 2011, 2 (7): 945-951.
45. Patel N, Panda S "Liposome drug delivery system: a critic review" *JPSBR*, 2012; 2 (4): 169-175.
46. Sirisha VNL, BhavaniHarika I, et al "Liposomes-the potential drug carriers" *J Pharm*, 2012, 2 (5): 26-38.
47. El-Nesr OH, Yahiya SA, et al "Effect of formulation design and freeze-drying on properties of fluconazole multilamellar liposomes" *SPJ*, 2010, 18: 217-224.
48. Sipai ABM, Vandana Y, et al "Liposomes: an overview" *JPSI*, 2012, 1 (1): 13-21.
49. Lopes LB, Scarpa MV, et al "Interaction of sodium diclofenac with freeze-dried soya phosphatidylcholine and unilamellar liposomes" *Braz J Pharm Sci*, 2006, 42 (4): 497-504.
50. Shivhare UD, Ambulkar DU, et al "Formulation and evaluation of pentoxifylline liposome formulation" *Dig J Nanomater Bios*, 2009, 4 (4): 857-862.
51. Perrie Y, Frederik PM et al "Liposome-mediated DNA vaccination: the effect of vesicle composition" *Vaccine*, 2001, 19: 3301-3310.
52. Traïkia M, Warschawski DE, et al "Formation of unilamellar vesicles by repetitive freeze-thaw cycles: characterization

by electron microscopy and ^{31}P-nuclear magnetic resonance" *Eur Biophys J*, 2000, 29: 184-195.
53. Shazly GA "Propranolol liposomes: formulation, characterization, and *in vitro* release" *J Optoelectron Biomed Mater*, 2013, 5 (1): 17-25.
54. Boltič Z, Petkovska M, et al "*In vitro* evaluation of the controlled release of antibiotics from liposomes" *Chem Ind*, 2003, 57 (12): 589-595.
55. Dua JS, Rana AC, et al "Liposome: methods of preparation and applications" *IJPSR*, 2012, 3 (2): 14-20.
56. Chang HI, Yeh MK "Clinical development of liposome-based drugs: formulation, characterization, and therapeutic efficacy" *Int J Nanomedicine*, 2012, 7: 49-60.
57. Cocero MJ, Martin A, et al "Encapsulation and co-precipitation processes with supercritical fluids: fundamentals and applications" *J Supercrit Fluid*, 2009, 47: 546-555.
58. Karn PR, Cho W, et al "Characterization and stability studies of a novel liposomal cyclosporin A prepared using the supercritical fluid method: comparison with the modified conventional Bangham method" *Int J Nanomedicine*, 2013, 8: 365-377.
59. Zhong J, Dai LC "Liposomal preparation by supercritical fluids technology" *Afr J Biotechnol*, 2011, 10 (73): 16406-16413.
60. Darani KK, Mozafari MR "Supercritical fluids technology in bioprocess industries: a review" *J Biochem Tech*, 2009, 2 (1): 144-152.
61. Reddy KH, Reddy MM "*In-vitro* formulation and evaluation of cefixime liposome formulation" *Int J Pharm Bio Sci*, 2012, 2 (2): 198-207.
62. Eldem T, Arican-Cellat N "High-performance liquid chromatographic determination of amphotericin B in a liposomal pharmaceutical product and validation of the assay" *J Chromatogr Sci*, 2000, 38: 338-344.
63. Wei GL, Xiao SH, et al "Validated HPLC assay of liposome-encapsulated and non-liposomal doxorubicin in plasma and its application to pharmacokinetic study" *AJPP*, 2010, 10 (3): 229-236.
64. Torres IMS, Bento EB, et al "Preparation, characterization and *in vitro* antimicrobial activity of liposomal ceftazidime and cefepime against *Pseudomonas Aeruginosa* strains" *Braz J Microbiol*, 2012, 43 (3): 984-992.
65. Phayre AN, Vanegas Farfano HM, et al "Effects of pH gradients on liposomal charge states examined by capillary electrophoresis" *Langmuir*, 2002, 18: 6499-6503.
66. Pysher MD, Hayes MA "Examination of the electrophoretic behavior of liposomes" *Langmuir*, 2004, 20: 4369-4375.
67. Nguyen TTTN, Østergaard J, et al "Investigation of a liposomal oxaliplatin drug formulation by capillary electrophoresis hyphenated to inductively coupled plasma mass spectrometry (CE-ICP-MS)" *Anal Bioanal Chem*, 2012, 402: 2131-2139.
68. Hautala JT, Wiedmer SK, et al "Anionic liposomes in capillary electrophoresis: effect of calcium on 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphatidylcholine/phosphatidylserine-coating in silica capillaries" *Anal Bioanal Chem*, 2004, 378: 1769-1776.
69. Shahi S, Sonwane U, et al "Design and development of diphenhydramine hydrochloride topical liposomal drug delivery system" *Int J Pharm Pharm Sci*, 2013; 5 (3): 534-542.
70. Al-Shdefat R, Yassin AEB, et al "Preparation and characterization of biodegradable paclitaxel loaded chitosan microparticles" *Dig J Nanomater Bios*, 2012, 7 (3): 1139-1147.
71. Simonzadeh N "An isocratic HPLC method for the simultaneous determination of cholesterol, cardiolipin, and DOPC in lyophilized lipids and liposomal formulations" *J Chromatogr Sci*, 2009, 47: 304-308.