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Techniques to Produce Liposomes for the Emerging Market of Nano-Based Formulations

Stéphanie Andrade; Maria João Ramalho; Joana Loureiro*; Maria Carmo Pereira*

LEPABE-Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal.

Corresponding Author: Joana A Loureiro & Maria C Pereira

LEPABE-Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal.

Tel: +351-22-508-4858 & +351-22-508-1590;

Email: joana.loureiro@fe.up.pt

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Abstract

Liposomes are one of the most popular drug delivery systems and have been used for clinical applications for almost three decades. Different techniques can be used to prepare and reduce the size of liposomes. The physicochemical properties of liposomes depend on the lipid composition and preparation method. Hence, these must be carefully thought during the liposomes' design stage. This chapter presents an overview of the most used methods for liposomes production and size reduction. The main advantages and limitations of each experimental procedure are here presented. The selection criteria for the lipid composition during liposome's design are also discussed, and future perspectives are presented.

Introduction

Nanomaterials for drug delivery application have dictated trends in the last decades due to their tremendous potential in enabling the therapeutic efficiency of drugs and selectively reaching the target tissues without toxicity for the nontarget organs. Nanomaterials exhibit unique physicochemical properties due to their small size and larger surface area that are advantageous for drug delivery. Valuable physicochemical properties such as enhanced stability, wide range of possible compositions, biocompatibility and biodegradability are crucial for the success of these nanosystems as Drug Delivery Systems (DDS).

Liposomes, first described by Bangham in 1964 [1], are one of the most popular DDS. Liposomes have received significant attention for several other biomedical applications such as imaging/diagnostic tools [2] and biomimetic membranes models [3,4]. Liposomes are spherical lipid vesicles composed of concentric bilayers of self-assemble phospholipids in aqueous medium [5]. They can be classified by their size and number of bilayers. Regarding the number of bilayers, liposomes can be divided in two categories, Multilamellar Lipid Vesicles (MLV) that are composed by many concentric lipid bilayers, and Uni-

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lamellar Lipid Vesicles (ULV). ULV can then be named by their size as Small (SUV), Large (LUV) and Giant (GUVs) Unilamellar Vesicles (figure 1) [6]. LUV are often preferred for drug delivery applications due to having a higher trapped volume that SUV which allows the loading of higher amounts of drugs than SUV. Also, when comparing to MLV, LUV require less quantity of lipids to entrap the same amount of drugs. However, SUV usually have a higher blood-circulation time duo to their smaller size [7]. Liposomes are advantageous for drug delivery due to their biodegradability, biocompatibility, high purity, ability to entrap both hydrophobic and/or hydrophilic molecules. While hydrophilic drugs are transported in the center of the liposome (aqueous compartment), hydrophobic drugs are transported in the bilayer [2].

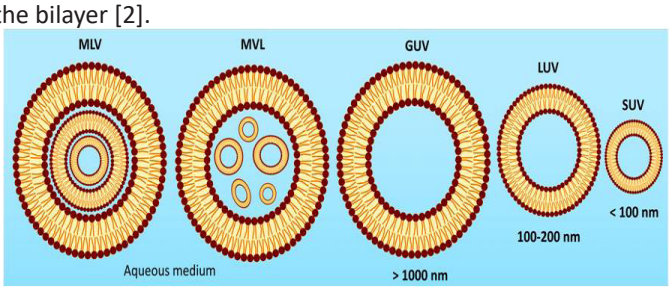

Figure 1: Schematic representation of the different types of liposomes: Multilamellar Vesicle (MLV), Multivesicular Liposome (MVL), Giant Unilamellar Vesicle (GUV), Large Unilamellar Vesicle (LUV) and Small Unilamellar Liposome Vesicle (SUV).

Liposomes were the first nanosystem successfully translated into the market for clinical application as DDS. For the first time in 1995, the medicine commercially called Doxil®, was used in the clinical to transport the drug doxorubicin in liposomes aiming to treat Kaposi's sarcoma [8]. After that formulation arriving to the market, the United States Food and Drug Administration (FDA) and European Medicines Agency (MDA) approved several other for clinical use.

The liposomes available in the market are used to transport drugs for the treatment of cancer, fungal diseases, pain management, viral vaccines, and can be also used for photodynamic therapy [9]. However, the main therapeutics are used for the cancer therapy (figure 2). The most common drugs encapsulated in the liposomes available in the market are doxorubicin, paclitaxel, and amphotericin B. Some of the most relevant liposomal formulations in the healthcare sector are Doxil®, Ambisome® and DepoDur™ (Table 1). Besides the liposomal formulation in the market, much more formulations are currently undergoing clinical trials [10].

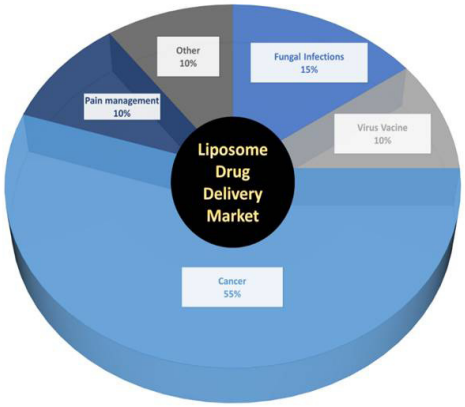

Figure 2: Liposomal products in the market covered by therapeutic areas.

Table 1: Some of the liposomes-based nanosystem used in clinical for the treatment of different types of diseases.

Type of Disease	Commercial name	Active compound
Cancer	Abraxane Doxil Evacet Lipo-Dox Myocet Marqibo Mepact Onivyde	Paclitaxel Doxorubicin Doxorubicin Doxorubicin Doxorubicin Vincristine sulfate *Mifamurtide* Irinotecan
Virus Vaccine	Epaxal Inflexal	Inactivated hepatitis A virus Influenza virus surface antigens
Fungal Infections	Abelcet AmBisome Amphocil	Amphotericin B Amphotericin B Amphotericin B
Pain Management	DepoDur Exparel	Morphine sulfate Bupivacaine

So, due to the enhanced benefits of the liposomes use in the clinical over the years, it is expected that the liposome drug delivery market substantially increases in the next few years. Some consulting companies estimate that till 2027 the market will double. In 2018, this market was estimated to round the US$ 3.6 Bn.

Nowadays, several strategies have been used to improve the liposomes' characteristics, such as surface modification to increase their bioavailability and surface decoration with targeting molecules to the efficient delivery of drugs in the required site of action [11]. The biophysical properties of liposomes significantly differ by varying the lipid composition and method of production [12]. Liposomes can be synthesized by a variety of experimental procedures and an overview of the most common methods for the preparation of liposomes are described in this chapter. Here, the criteria for choosing the lipid composition during liposome's design are also discussed.

Selection of phospholipids for the design

Conventional liposomes composed of neutral and/or anionic lipids were the first generation of lipid vesicles to be created and used in the pharmaceutical industry. However, liposomes can be prepared with unlimited number of lipids and lipid combinations. The choice of lipids, and consequently nature and size of the polar head group, length and degree of unsaturation of the acyl chain will affect the properties of the prepared liposomes, such as size, surface charge, stability, encapsulation efficiency and others [13]. Liposomes with small sizes, high encapsulation efficiency and colloid stability are the main demand for drug delivery purposes. Size and surface charge are key features to drug delivery and different phospholipids present advantages and disadvantages depending on the intended application. Liposomes' ability to cross the biological barriers and be uptaken by target cells is mainly dependent on liposomes' size and charge. For example, size regulates the biodistribution and clearance of liposomes. Evidence suggest that nanoparticles below 10 nm undergo renal clearance [14], and large liposomes (>200 nm) are quickly cleared from the blood circulation and more difficult to permeate cell membranes [15]. The surface charge of nanocarriers is also closely related to their biological fate since electrostatic interactions regulate several phenomena such as cell uptake and cell binding. Thus, during the design of liposomes,

their composition must be carefully thought out.

Polar head groups' charge and size

The polar head group is a major factor influencing liposomes' properties, since the overall net charge of the liposomes depends on the choice of used phospholipids. For example, the entrapment of hydrophilic molecules depends on the surface charge of the liposomes, with the encapsulation efficiency being lower for neutral liposomes than for charged ones. Cationic liposomes also exhibit higher encapsulation of hydrophilic drugs than negatively charged liposomes [14].

Surface charge is also a main regulator of liposomes' stability that is mainly related to changes in liposomes' size due to aggregation/fusion of vesicles and leakage of the entrapped drug. Surface charge confers electrostatic stabilization, thus charged liposomes offer more stability than neutral liposomes [15]. However, as serum proteins can bind to charged liposomes and inactivate them and/or alter their biodistribution and stability, neutral liposomes can also offer an advantage [16]. Several works reported that cationic liposomes are able to recruit serum proteins *in vivo* that can confer a natural targeting ability [17]. Also, cationic liposomes are usually reported as being more likely to penetrate biological barriers and easily internalized by cells due to their ability to interact with the negatively charged cell membranes [18]. Thus, positively charged liposomes are usually preferred for drug delivery due to their high affinity and residence time with the target cells [19]. These are widely popular for gene therapy due to their ability to form cationic liposomes-nucleic acid complexes, known as lipoplexes [20,21]. However, some studies showed that anionic liposomes exhibit a higher cerebral uptake than cationic or neutral [15]. Though, the surface charge of liposomes must be carefully thought out since the positive charged liposomes are usually associated with higher toxicity. Cationic liposomes exhibit toxic effects which deters their clinical use due to the destabilization of the cell membranes [22], and production of reactive oxygen species [23,24]. Negatively charged liposomes also offer other advantages over cationic ones, such as higher stability in solution upon storage [19].

The influence of the polar head groups on liposomes properties is not only duo to their net charge but also due to their size. For example, liposomes' physical stability is also controlled by the size of head groups. Studies reported that liposomes containing phospholipids with larger head-groups exhibit enhanced stability due to an increased steric effect [16].

Alkyl chain length

The length of the alkyl chain influences the properties of the produced liposomes. For example, the size is dependent on the alkyl chain length. Although liposomes' sizes are mainly regulated by the method of production, long chain fatty acids usually yield larger liposomes, exhibiting a higher packing parameter and decreased fluidity, which is favorable for the formation of smaller vesicles [25].

The length of the hydrocarbon chain of phospholipids also affects the main phase transition temperature (Tm) that is a crucial factor controlling liposomal stability. Evidences have shown that the Tm increases with the length of the phospholipids' acyl chain, increasing the liposomes' stability [16].

The entrapment of hydrophobic drugs is also affected by the alkyl chain length. While for hydrophilic molecules, the entrapment efficiency is more dependent on the surface charge of liposomes, for hydrophobic drugs drug encapsulation depends mainly on the length and degree of unsaturation of the alkyl chain. The encapsulation efficiency of hydrophobic drugs increases with the length of the chain, due to the more available space for drug accommodation within the bilayer [26].

Alkyl chain degree of unsaturation

The choice of alkyl chain unsaturation degree of phospholipids is crucial for liposomes features. For example, the fluidity of the bilayer is modulated by the degree of the saturation of the hydrocarbon chain, increasing with the degree of unsaturation. As consequence, drug encapsulation increases with the degree of unsaturation of the alkyl chain, with liposomes composed of unsaturated phospholipids exhibiting higher encapsulation efficiency values. A the fluidity of the bilayer increases with the degree of unsaturation of the phospholipids chains, the available space for drug accommodation increases [27]. Also, saturated phospholipids usually confer higher stability to the liposomes [28].

Other lipid components

Besides phospholipids other lipid molecules are usually used for liposomes production as cholesterol, sphingolipids (namely glycolipids and sphingomyelin), polymers such as polyethylene glycol. Cholesterol is one of the most used molecules for the formulation of conventional liposomes, also known as first-generation liposomes, to regulate the physicochemical properties of the nanocarriers. This lipid modulates the rigidity, thickness, and fluidity of the bilayer, increasing the liposomes' stability. Some evidences show that cholesterol is able to increase the blood circulation time of nanosystems [29]. Although, its use presents some disadvantages. For example, the encapsulation efficiency of hydrophobic drugs decreases with the increase in cholesterol content, due to an increase in the rigidity and a competition for the available accommodation space [30].

Research on liposome technology has progressed to second-generation liposomes, and PEG is used to produce stealth liposomes with extend blood-circulation time able to avoid recognition by the mononuclear phagocyte system [31].

Methods of liposomes preparation

The preparation of liposomes can be achieved using a variety of techniques. Here, the most common methodologies used to prepare LUV are described, and the main advantages and disadvantages presented, as summarized in Figure 3.

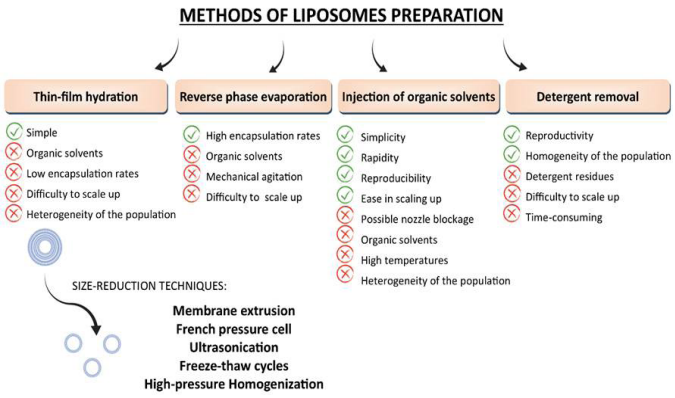

Figure 3: Main techniques of liposomes preparation, with respective advantages and disadvantages.

Thin-film hydration

The thin-film hydration, also known Bangham method, is the most common and simple technique used to prepare liposomes. In a first stage, lipids are dissolved in an organic solvent such as chloroform, dichloromethane, ethanol or a mixture of chloroform and methanol. Then, the organic solvent is removed by evaporation producing a thin lipid film. The dry film is hydrated with an aqueous solution and vortexed, forming lipid vesicles [32]. This technique presents some disadvantages such as the use of organic solvents, the relatively low encapsulation efficiencies, and the difficulty to scale up from the laboratory level to the industrial production. Moreover, the obtained dispersion contains a population of MLV heterogeneous in size and shape [33]. Therefore, further methodologies are required to reduce the liposomes' size and obtain homogeneous populations of LUV. The main size-reduction techniques are described below.

A. Ultrasonication

Ultrasound is the most used common method for the reduction of liposomes' size. Through this technique it is possible to disrupt MLV previously produced to obtain SUV and LUV with diameters ranging from few nanometers to a hundred nanometers [34]. Depending on the ultrasound's frequency, time of sonication, volume, lipids concentration and lipids nature, it is possible to obtain liposomes with different sizes [35]. Normally, the sonication occurs between 5 and 15 minutes and should be performed above the lipid Tm. This period could be continuous or interspersed and could be done using a bath or a probe tip sonicator. This method is fast and unexpensive. However, sometimes, due to the high energy delivery to the lipid suspension, it can suffer from overheating. That phenomenon can cause lipid or drug degradation (when the objective is to encapsulate drugs). Also, in the case of the probe tip sonicator, the tips can release small particles of titanium in the lipid suspension, contaminating it.

The reproducibility of size is also a disadvantage since it is almost impossible to exactly reproduce the sonication conditions.

B. Membrane extrusion

Extrusion is another frequently used technique to reduce the size of MLV. Here, pressure is applied to the MLV suspension forcing its repeated passage through polycarbonate membranes with a known pore size to produce LUV with a mean diameter ranging from 110 to 150 nm. Compared to other size reducing techniques, the extrusion method produces relatively more homogenous particles [36]. The extrusion process must be performed at a temperature above the lipid Tm since the lipid membrane is in a fluid and flexible state, which facilitates the passage of liposomes through the pores. This also improves the encapsulation efficiency of drug-loaded liposomes. Other main advantage of the extrusion is the reproducibility between batches. However, it also presents some limitations such as the difficulty to scale-up, fragility of the membranes, possibility of membranes clogging, the strict control of the pressure applied, and the time consumed [33]. Besides, the total pore surface area corresponds only to 20% of the total surface area of the membrane, which restricts the surface area available for extrusion, thus limiting the total throughput (about 70%) [36].

C. French pressure cell

Like the extrusion method, the French pressure cell technique also involves the extrusion of MLVs, but here through a small orifice by applying high pressures, producing LUVs. One passage of the MLV suspension typically results in 70% of LUVs; a second passage increases this value to 90%. This method is simple, quick, and reproducible. However, the limited working volumes (maximum of 50 mL) represents a disadvantage. Liposomes produced by this method are larger than those obtained by sonication; however more stable [37].

D. Freeze-thaw cycles

Freeze-thaw cycles are commonly used to reduce the liposomes' size and increase the encapsulation efficiency of drugs into lipid vesicles. This method consists in submerging the suspension that contain the MLV in a liquid at very low temperature (typically liquid nitrogen) followed by submerging in high temperature bath [38]. So, a large gradient of temperature occurs. The freeze-thaw cycles cause the abrupt disruption and quickly reassembly of successively smaller vesicles. The lamellarity of MLV decreases through the cycles and the drug encapsulation rate increases. To successfully increase the drug encapsulation, multiple freeze-thawing cycles at temperatures above lipid Tm should be performed. The freeze-thaw method depends on the ionic strength of the medium and the phospholipid concentration. The main limitation of this procedure is that some drugs cannot be exposed at that variation of temperatures since they can be degraded. Also, high lipid concentrations cannot be used.

E. High-pressure homogenization

High-pressure Homogenization (HPH) is commonly used to produce liposomes in large-scale. This method is accomplished by forcing the MLV suspension to a high pressure up to 400 MPa [39]. Through the HPH, it is possible to rapidly reduce the MLV' size and lamellarity in order to obtain LUVs [38]. Also, with this method is possible to reduce/prevent the number of vesicle aggregations. In the first cycle of HPH already occurs a significant decrease of size, leading to the formation of LUV. This process appears to be fast. In this production process, the liposomes' size could be influenced by several factors, such as homogeneity of the initial product, processing pressures and number of cycles, ionic strength of the medium, temperature and lipid composition and loading content. Also, the homogenizing chamber design could affect the success of the procedure, mainly related with the distribution of pressure on the vesicles. Usually, the liposomes' size decreases with the increasing in the inlet pressure.

One disadvantage related with this procedure is the drugs' degradation caused by the strong cavitation and shear forces formed. Other disadvantage is related to the homogenizer erosion, that can contaminate the liposomes suspension.

Reverse phase evaporation

The reverse phase evaporation is a simple alternative technique to the lipid film hydration. Here, phospholipids are dissolved in a water-immiscible organic solvent. Then, a water-in-oil emulsion is created by brief sonication, and the organic solvents evaporated under reduced pressure to produce a semi solid gel. The gel is subject to a vigorous mechanical agitation to

reverse the water-in-oil into an oil-in-water emulsion containing LUVs and MLVs. Lower concentrations of phospholipids yield higher amounts of LUVs compared to MLVs. This process ensures high encapsulation rates due to the large volume: lipid ratio [37]. However, the use of high amounts of organic solvents, mechanical agitation, the possibility of the product to contain solvent residues and difficulty to scale up remains its major limitations [40].

Injection of organic solvents

The injection of organic solvents is widely used to produce liposomes. Here, phospholipids dissolved in an organic solvent, such as ethanol or ether, are gradually injected in a warm aqueous solution of the molecule to be encapsulated, spontaneously forming LUVs and SUVs. When ethanol is injected and dissolved in water below a critical concentration, the phospholipids are forced to self-assemble in the aqueous phase and form liposomes. In contrast, the injection of ether in an aqueous solution leads to the production of liposomes by evaporating the organic solvent under vacuum. Higher concentration of phospholipids leads to an increase on the size, polydispersity and lamellarity of liposomes. The main drawbacks of this technique are the heterogeneity of the population when compared to other methods [37], with the size of liposomes ranging from 30 to 200 nm, possible nozzle blockage. Also the exposure of the compounds to be encapsulated to organic solvents and high temperatures can affect the stability and biocompatibility of the liposomes [6]. However, the simplicity, rapidity, reproducibility, and ease in scaling up this technique makes it a promising methodology for industry applications [33].

Detergent removal

Detergent removal is another commonly employed methodology to prepare LUVs. Here, phospholipids are solubilized in an aqueous solution of detergent micelles. Removing the detergent by dialysis or column chromatography induces the fusion of the phospholipid micelles and consequent formation of LUVs. This technique exhibits high reproductivity and produces LUVs homogeneous in size, with mean diameters ranging from 100 to 200 nm. The homogeneity of liposomes is only guaranteed when reduced lipid concentrations are used; concentrations up to 2% of lipid (w/w) increase the liposomes' size distribution and lamellarity. Besides, detergent removal method presents other weaknesses including the presence of detergent residues within the lipid vesicles, the difficulty to scale up and being a time-consuming technique [41].

Concluding remarks and future perspectives

The development of effective DDS able to reduce side effects of drugs while enhancing their therapeutic activity are one of the major concerns of the pharmaceutical industry and scientific community. Liposomes are one of the most successful delivery systems, with some liposomal formulations already available on the market but with the majority remaining in the pipeline.

Since their discovery in the mid-1960's, liposomes have been intensively studied in terms of composition, production techniques and applications. The choice of the lipid composition as well as the method to be used to produce liposomes seem to be the main factors influencing the efficacy of formulations, since they affect the drug encapsulation, release and ADME-Tox (absorption, distribution, metabolism, excretion, and toxicity) properties.

The traditional methodologies used to prepare and reduce the lamellarity and size of liposomes remain popular since they are relatively simple and do not involve sophisticated equipment. However, most of them are difficult to scale up from the laboratory to the industrial manufacture. Besides, all the conventional methods here described require the use of organic solvents or detergents. These can affect the membrane's properties, reduce the liposomes' stability, and represent a possible risk to health. Thus, novel methodologies to overcome the drawbacks of the conventional techniques are emerging over the last decade, although they are still at an early stage of the optimization process. The recent advances mainly focus on large-scale production of liposomes, absence of organic reagents and high encapsulation rates. Regrettably, no modern technique has been accepted so far as a new standard method of liposomes production. Future efforts should be made to expand the commercial availability of liposomal formulations.

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