Design of liposomes as drug delivery system for therapeutic applications

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Abstract

Liposomes are spherical vesicles consisting of one or more concentric phospholipid bilayers enclosing an aqueous core. Being both nontoxic and biodegradable, liposomes represent a powerful delivery system for several drugs. They have improved the therapeutic efficacy of drugs through stabilizing compounds, overcoming obstacles to cellular and tissue uptake and increasing drug biodistribution to target sites *in vivo*, while minimizing systemic toxicity. This review offers an overview of liposomes, thought the exploration of their key fundamentals. Initially, the main design aspects to obtain a successful liposomal formulation were addressed, following the techniques for liposome production and drug loading. Before application, liposomes required an extensive characterization to assurance *in vitro* and *in vivo* performance. Thus, several properties to characterize liposomes were explored, such as size, polydispersity index, zeta potential, shape, lamellarity, phase behavior, encapsulation efficiency, and *in vitro* drug release. Topics related with liposomal functionalization and effective targeting strategies were also addressed, as well as stability and some limitations of liposomes. Finally, this review intends to explore the current market liposomes used as a drug delivery system in different therapeutic applications.

Keywords: Liposomes; Drug delivery; Liposome production; Liposome characterization; Market liposomes.

Abbreviations

AFM, Atomic force microscopy; AIDS, Acquired immunodeficiency syndrome; ASES, Aerosol solvent extraction systems; CH, Cholesterol; CO_2, Carbon dioxide; Cryo-TEM, Cryogenic – TEM; DLS, Dynamic light scattering; DOPC, 1,2-dioleoyl-snglycero-3-phosphocholine; DOPE, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine; DPPC, 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine; DPPG, 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol; DSC, Differential scanning calorimetry; DSPC, 1,2-Distearoyl-sn-glycero-3-phosphocholine; DSPE, 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine; DSPG, 1,2-Distearoyl-sn-glycero-3-phosphoglycerol; EDTA, Ethylenediaminetetraacetic acid; EE, Encapsulation efficiency; EPR, Enhanced permeability and retention; ESR, Electron spin resonance; FDA, Food and Drug Administration; FFF, Field-flow fractionation; FTIR, Fourier transform infrared spectroscopy; GAS, Gas anti-solvent; GC-MS, Gas chromatography–mass spectrometry; HPLC, High-performance liquid chromatography; LC-MS, Liquid chromatography–mass spectrometry; LDE, Laser Doppler electrophoresis; LUV, Large Unilamellar Vesicles; MCL, Multicompartment liposome; MLV, Multilamellar Vesicles; MPS, Mononuclear phagocytic system; MVV, Multi Vesicular Vesicles; NMR, Nuclear magnetic resonance; NTA, Nanoparticle tracking analysis; PA, Phosphatidic acid; PC, Phosphatidylcholine; PCS, Photon correlation spectroscopy; PDI, Polydispersity index; PE, Phosphatidylethanolamine; PEG, Polyethylene glycol; PG, Phosphatidylglycerol; PI, Phosphatidylinositol; PS, Phosphatidylserine; RES, Reticuloendothelial system; RESS,

Rapid expansion of supercritical solutions; SAS, Supercritical anti-solvent; SAXS, Small-angle X-ray scattering; SCF, Supercritical fluid; SCRPE, Supercritical reverse-phase evaporation; SEC, Size exclusion chromatography; SUV, Small Unilamellar Vesicles; Tc, Transition temperature of phospholipids; TEM, Transmission electron microscopy; TGA, Thermogravimetric analysis; UPLC, Ultra-performance liquid chromatography; UV–Vis, Ultraviolet-visible; XRD, X-ray diffraction.

1. Introduction

Liposomes were initial discovered in the 1960s by the British hematologist Dr. Alec D. Bangham and collaborators at the Babraham Institute, University of Cambridge, and the first report published in 1964 (Bangham and Horne, 1964). Liposomes are defined as a colloidal spherical structure formed by self-assembly of amphiphilic lipid molecules in solution, such as phospholipids (Sebaaly et al., 2016). Liposomal membrane can be composed of one or more lipid bilayers (lamellas) organized around an internal aqueous core, with the polar head groups oriented to the inner and outer aqueous phase (Nisini et al., 2018). This organized structure offers to liposomes the unique ability to load and deliver molecules with different solubility. Hydrophilic molecules in the internal aqueous core, hydrophobic molecules into the lipid bilayer and amphiphilic molecules at the water/lipid bilayer interface (Fig. 1) (Laouini et al., 2012a).

Fig 1. Representation of the general structure of liposomes.

To date, liposomes have been investigated in several pharmaceutical research as drug delivery systems and continue to constitute an intense field of research (Bozzuto and

Molinari, 2015). Liposomes are considered a powerful drug delivery systems due to their structural versatility as well as their biocompatibility, biodegradability, non-toxic and non-immunogenicity nature (Mathiyazhakan et al., 2018). The amphiphilic character of phospholipids in solution mimic natural cell membranes, allowing excellent interactions between liposomes and mammalian cell membranes promoting an efficient cellular uptake (He et al., 2019). Additional advantages of liposomes include their ability to carry large drug payloads, capacity for self-assembly and a wide range of physicochemical and biophysical properties that can be modified to control their biological characteristics (Sercombe et al., 2015).

Liposomes as a drug delivery system have improved therapies for a range of biomedical applications by stabilizing therapeutic compounds, overcoming obstacles to cellular and tissue uptake, and improving bio-distribution of compounds to target sites *in vivo* (Ding et al., 2006; Hua and Wu, 2013). The drug loaded into liposome is protected against physiologically occurring events, such as enzymatic degradation, chemical and immunologic inactivation and fast plasma clearance, contributing to improve and extension of its action. Since the drug is inside the liposome, there is a minimization of its exposure to healthy tissue, reducing the undesirable side effects compared with the free drug form (Bozzuto and Molinari, 2015).

2. Design of liposomes

A suitable liposomal formulation can be achieved by choosing an adequate liposome composition, functionalization and even a targeting strategy, as developed deeper in the following sections. The selection of phospholipids, head group and chain length, as well as the ratio of liposomes components are crucial features to determine safety, stability, and efficiency of liposomes (Kapoor et al., 2017). Moreover, the ability of liposomes as

drug delivery system can be affected by the number and rigidity of lipid bilayers, size, surface charge, lipid organization and surface modification (Euliss et al., 2006; Sebaaly et al., 2016).

2.1. Liposome components and properties

The main component of liposomes are glycerophospholipids, which are amphiphilic lipids composed of a glycerol molecule bound to a phosphate group and to two fatty acid chains that may be saturated or unsaturated (Pinot et al., 2014). The phosphate group can be also bonded to another organic molecule (Beltrán-Gracia et al., 2019; Monteiro et al., 2014a). According to this organic group, natural phospholipids are classified as phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylserine (PS) (Tsuji et al., 2019). Glycerophospholipids that are responsible to form liposomes can be divided in two different forms: natural and synthetic. The most natural phospholipids used to produce liposomes are PC and PE, that are abundant phosphatides in plants and animals (Antimisiaris et al., 2007). The main sources of natural phospholipids are egg yolk or soya bean. Synthetic phospholipids are produced from natural lipids. Modification in head groups, aliphatic chains and alcohols of natural phospholipids creates a variety of synthetic phospholipids, that have proved to be more stable. Some examples of phospholipids in the synthetic form are 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-snglycero-3-phosphocholine (DOPC), 1,2-Distearoyl-sn-glycero-3-phosphoglycerol (DSPG), 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine, (DOPE) and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) (Monteiro et al., 2014a).

In an aqueous environment, phospholipids have a strong ability to form stable bilayers due to their amphipathic character. Therefore, liposomes are formed by hydrophilic interactions between polar head groups, van der Waals forces between hydrocarbon chains (keep the long hydrocarbon tails together) and hydrogen bonds with water molecules. Hydrophobic chains are repelled by water molecules and spontaneously occurs the self-assembly of liposomes in a closed bilayer (Frézard, 1999; Monteiro et al., 2014a). Liposomes can be a combination of two or more phospholipids and consisted of single or multiple lipid bilayers. Depending on the head of the phospholipids, liposomes can acquire positive, negative, or neutral charges (Lombardo et al., 2016). The final liposomal properties are influenced by the structure and characteristics of phospholipids. Liposomes can achieve different functionality with variations in head groups, aliphatic chains and in the saturation of fatty acids (Liu et al., 2019). The stability of liposomes can be promoted using phospholipids with longer tails, and low degrees of tail unsaturation and ether linkages. Phospholipids with longer saturated hydrocarbon chains have higher ability to interact each other and to form rigidly ordered bilayer structures. Otherwise, phospholipids with shorter unsaturated hydrocarbon chains that form liposomes with fluid and disordered bilayers (Kapoor et al., 2017; Rawicz et al., 2000).

In addition to phospholipids, there are more liposomal components that can enhance the stability of liposomes such as cholesterol (CH), glycols including propylene glycol and polyethylene glycol (PEG) and even polymers as chitosan. These components can have pronounced effects on healthy tissues and cells, as well as activate or suppress the immune system (Inglut et al., 2020). The incorporation of CH to the lipid bilayer of liposomes can influence the bilayer fluidity and rigidity reducing their permeability and increased their *in vitro* and *in vivo* stability. CH, as a hydrophobic molecule, induces a dense packing of phospholipids and inhibits the interactions in the lipid chains by

intercalating between them, promoting the stabilization of the liposomes membrane (Bozzuto and Molinari, 2015; Lee et al., 2005; Sharifi et al., 2019). CH molecule accommodates itself among the phospholipids with its hydroxyl group close to the hydrophilic region, and its aromatic rings parallel to the fatty acid chains into the lipid bilayer (Beltrán-Gracia et al., 2019). CH is crucial for the structural stability of liposomes, in their absence liposomes can interact with proteins (albumin, transferrin, macroglobulin and high-density lipoproteins). These interactions destabilize the structure of the liposomal membrane and consequently decreases their performance as drug delivery system (Lu et al., 2013; Maranhão et al., 2017; Yingchoncharoen et al., 2016).

Another way to achieve the liposomal structure modification is the incorporation of glycols. Phospholipid vesicles with propylene glycol have been advocated as flexible lipid vesicles in order to obtain delivery systems for enhanced the skin delivery of drugs (Elmoslemany et al., 2012; Manconi et al., 2009). The use of different PEGs on the surface of liposomes can be a good approach to prolong blood circulation half-life from few minutes (conventional liposomes) to several hours (stealth liposomes, also called PEGylated liposomes) (Beltrán-Gracia et al., 2019). Indeed, one of the major drawbacks of conventional liposomes are their rapid clearance from the bloodstream and end up in organs and tissues in the reticuloendothelial system (RES) such as liver and spleen (Lee and Thompson, 2017). The increase in circulation lifetime of the liposomes promoted by PEG has been found to depend on both the amount of grafted PEG and the molecular weight or length of the polymer (Allen et al., 1991). Usually, the longer-chain PEGs have produced the greatest improvements in blood circulation time (Immordino et al., 2006). It was reported that blood levels were higher for PEGylated liposomes with longer molecular weight (PEG 1900 and PEG 5000) compared to PEGylated liposomes containing a shorter chain (PEG 750 and PEG 120) (Allen et al., 1991). The conformation

of the PEG polymers on the surface of the liposomes is determined by the PEG molecular weight and the PEG surface density, and can be mushroom (low concentration) or brush (high concentration) regime (Perry et al., 2012). The increased in PEG concentration from 5% to 10% (molar ratio) showed a clearly improved in the stealth degree of the liposomes. Liposomes that have a higher concentration of PEG (brush regime) are more resistant to phagocytosis and poorly activate the human complement system (Nogueira et al., 2013).

The surface properties of liposomes improved by PEG are associated to a camouflaged effect, mimicking water-like structures, providing a steric barrier that prevents the adsorption of proteins in liposome surface and avoiding their recognition by macrophages of the mononuclear phagocytic system (MPS) that otherwise leads to a rapid liposome clearance (Kapoor et al., 2017; Monteiro et al., 2014a). Polymers such as chitosan is also used for the modification of the liposomal surface, leading to a protective shell on the liposome surface, mainly for the oral delivery of drugs (Caddeo et al., 2017; Henriksen et al., 1994). Besides the incorporation of the components described below on their composition, liposomes can be functionalized with specific ligands to improved their ability as drug delivery system, leads to a new category of liposomes called targeted liposomes. A more detailed description of the different types of liposomes will be reported below.

2.1.1. Phase transition temperature

Another important parameter that can affect the fluidity of the lipids within the bilayer is the transition temperature of phospholipids (Tc), which refers to the temperature at which phospholipids transit from gel to liquid crystalline phase (Zamani et al., 2018). The Tc depends on the length of the fatty acid chains, the degree of saturation of the hydrocarbon chains, the ionic strength of the suspension medium and the nature of the polar head group

(Hussain et al., 2017; Maurer et al., 2001). Lipid bilayers composed of phospholipids with long and saturated hydrocarbon chains should be rigid and less permeable, due the interactions between the chains are stronger, resulting in a higher Tc. Thus, hydrophobic interactions are stronger when the saturated hydrocarbon tails increase in length (Akbarzadeh et al., 2013). At a temperature lower than Tc, the phospholipids are in gel phase and presenting low fluidity and low permeability, individual molecules within the bilayer move gently. At a temperature higher than Tc, the phospholipids are in a liquid crystalline phase and having a high fluidity and usually relatively low permeability, individual molecules within the lipid bilayer move quickly. At a temperature around Tc, the liposome bilayer increases significantly the permeability due the presence of highly permeable interfacial regions between coexisting gel and liquid crystalline phase domains (Beltrán-Gracia et al., 2019; Collier and Messersmith, 2001).

2.2. Liposome structure

According to their structure, liposomes are classified centered on the number of lipid bilayers (lamellae) and on the vesicle size (Fig. 2). Based on their lamellarity, liposomes can be classified as unilamellar (ULV, all size range), multilamellar (MLV, >500 nm) and multivesicular (MVV, >1000 nm) vesicles (Akbarzadeh et al., 2013; Emami et al., 2016). ULV can also be divided by their size into three categories, small unilamellar vesicles (SUVs, 20 – 100 nm), large unilamellar vesicles (LUVs, >100 nm) and giant unilamellar vesicles (GUVs, >1000 nm). ULVs are characterized by the presence of a single bilayer, with more ability for the encapsulation of hydrophilic compounds. MLVs present two or more concentric lipid bilayers organized by an onion like structure, favorably for the encapsulation of lipophilic compounds. MVVs include several small non-concentric vesicles entrapped within a single lipid bilayer and are ideally suited for

the encapsulation of large volume of hydrophilic material (Emami et al., 2016; Maherani et al., 2011). In addition to the vesicle size, the number of lamellae also affect the amount of certain compound to be encapsulated in liposomes (Akbarzadeh et al., 2013; Olusanya et al., 2018). Alternatively, an innovative vesicle-type formulation is the multicompartment liposome (MCL). The MCL is structurally composed of two different types of vesicles connected through a tight bilayer interface and are developed as single-vehicle delivery systems for combinatory compounds (Al-Jamal and Kostarelos, 2007; Catalan-Latorre et al., 2016).

Fig. 2. Liposomal classification based on lamellarity and size. SUV (Small Unilamellar Vesicles), LUV (Large Unilamellar Vesicles), MLV (Multilamellar Vesicles) and MVV (Multi Vesicular Vesicles).

3. Methods for liposome production and drug loading

There are a great variety of techniques for liposome production, including the liposomal formulation methods itself and the size reduction methods. The different techniques can influence the final properties of liposomes, such as size, lamellarity, and encapsulation efficiency (EE) (Pattni et al., 2015). The methods to produce liposomal formulations can be categorized as conventional or novel. In the following section will be explored some of these methods.

3.1. Conventional methods

Despite the vast gamma of conventional methods applied in liposome preparation, the most common used are the thin film hydration, reverse phase evaporation, solvent injection, and detergent removal method (Karn et al., 2013; Meure et al., 2008). These

methods involve the following basic stages: (i) lipids dissolved in organic solvents, (ii) removal of organic solvent, (iii) purifying and isolation of liposomes and (iv) analysis of final liposomes (Akbarzadeh et al., 2013).

3.1.1. Thin film hydration

The thin film hydration method, also known as the Bangham method, was the first described production process used in liposome technology (Bangham et al., 1967). In this simple method, lipids are initially dissolved in an organic solvent, generally chloroform, ether or methanol, and dried down to form a thin lipid film in a round-bottom flask by organic solvent evaporation. The obtained thin lipid film is hydrated using aqueous solvent and the liposomes are formed. Depending on hydration conditions, this method can create liposomes with different structural organization. A vigorous shaking at hydration process form MLVs with heterogeneous size, while a gentle hydration of the lipid film generates GUVs (Isalomboto Nkanga et al., 2019; Monteiro et al., 2014b). The main drawbacks of this method are the production of larger and heterogeneous liposomes, low entrapment ability, difficult to complete removal of organic solvent and to scale-up (Meure et al., 2008).

3.1.2. Reverse phase evaporation

One alternative method to prepare liposomes is the reverse phase evaporation. The initial procedure is the same of thin film hydration. Phospholipids are dissolved in an organic solvent to form a film and then the solvent are removed by evaporation. The film is re-dissolved in an organic solvent (typically, diethyl ether and/or isopropyl ether), followed by the addition of an aqueous phase, resulting in the formation of an oil-in-water emulsion (Pattni et al., 2015). The mixture is sonicated to produce inverted micelles, forming a

homogeneous emulsion. The final evaporation of the organic solvent under reduced pressure form a viscous gel, that results subsequently into a liposomal suspension (Akbarzadeh et al., 2013; Maherani et al., 2011). The advantage of this method is that permits a high EE (Monteiro et al., 2014b; Wagner and Vorauer-Uhl, 2011). The disadvantages include the exposure of the compounds to be encapsulated to sonication conditions and even the organic solvents (Antimisiaris et al., 2007). This method is also described as time-consuming (Meure et al., 2008).

3.1.3. Solvent injection techniques

Liposomes can also be prepared by the solvent injection technique. This method involves the quick injection of the lipids, dissolved in an organic solvent (ethanol or ether), into an aqueous medium, resulting in liposomes formation (William et al., 2020). The ethanol injection method is usual in liposomes production due its simplicity, reproducibility, fast implementation, easy scale-up and not cause lipid degradation or oxidative alterations (Justo and Moraes, 2010). Ethanol has also the additional benefit to be an acceptable solvent for *in vivo* drug delivery applications, at lower concentration, according to the European pharmacopoeia (Marasini et al., 2017). Despite all the benefits, poor solubility of some lipids in ethanol, heterogeneity of liposomes when the agitation fail, very low EE of hydrophilic compounds and incomplete removal of ethanol from the liposomes, are the most concerns about this method (Çağdaş et al., 2014; Maherani et al., 2011). Numerous parameters can be altered to control the particle size and EE obtained by ethanol injection method, such as lipid nature, lipid concentration in ethanol, drug to lipid ratio, diameter of injection orifice and injection rate (Maherani et al., 2011; Wagner and Vorauer-Uhl, 2011).

3.1.4. Detergent removal

The detergent removal method is another known technique to produce liposomes. In this method, phospholipids are solubilized with detergents at critical micelle concentrations (Isalomboto Nkanga et al., 2019). Upon detergent removal, by column chromatography or dialysis bags, and with an adequate aqueous medium, phospholipids molecules self-assemble into liposomes (Akbarzadeh et al., 2013; Pattni et al., 2015). Numerous parameters can influence the size and homogeneity of the liposomes produced by this method, including initial ratio of phospholipids to detergents and rate of detergent elimination (Maherani et al., 2011; Wagner and Vorauer-Uhl, 2011). The drawbacks of detergent removal method can be the presence of impurities in the final liposomal formulation, possible interaction between the detergent and the encapsulated compound and the fact of this technique to be very time-consuming (Meure et al., 2008; Schubert, 2003).

3.2. Size reduction techniques

Liposomes produced by most of the previous methods requires additional techniques to reduce their size, such as sonication, homogenization or extrusion (Kraft et al., 2014). There are two different sonication techniques that can be used to control the size of liposomes, a bath and a probe sonication (Akbarzadeh et al., 2013). The sonication process may have disadvantages as the difficult to provide identical ultrasonic energy in a large volume of liposomal suspension (scale-up) and potential metal contamination from the probe tip. Furthermore, there is possible risk of degradation on phospholipids and even on compound to be encapsulated, as well as low EE (Batzri and Korn, 1973; Tejera-Garcia et al., 2011). In homogenization techniques, liposomes can be forced to pass within an orifice through under high pressure to reduce their size, resulting in a

concept of high-velocity collision. Several techniques can be included in this category of size reduction, such as microfluidization, high-pressure homogenization, and shear force-induced homogenization processes (Wagner and Vorauer-Uhl, 2011). Another technique of reducing the size of liposomes is the extrusion process. After their formation, the liposomes pass several times (extrusion cycles) through a membrane of defined pore size, normally a polycarbonate filter, to uniform size distribution (Meure et al., 2008; Olson et al., 1979). This process requires much lower pressure and less volume of liposomal suspension compared with homogenizers (Kraft et al., 2014).

3.3. Novel methods

The novel methods of liposome preparation are being investigated mainly to facilitate the scale-up for industrial production and to be applied to a wide range of phospholipids and drugs (Pattni et al., 2015). There are novel methods based on the modification or improvement of conventional methods, such as cross-flow injection (Wagner) method (Wagner et al., 2002; Wagner and Vorauer-Uhl, 2011) and membrane contractor technology, both modified/improved of ethanol injection method (Charcosset et al., 2005; Patil and Jadhav, 2014). The improved of detergent removal technique designs the cross-flow filtration method (Karn and Hwang, 2015; Peschka et al., 1998). The direct hydration of lipid components following the sonication process also represent an easy method avoiding the use of dissipative steps (Manca et al., 2013; Manconi et al., 2003; Manconia et al., 2009). Furthermore, the use of supercritical fluid (SCF) methods has been explored in liposomes production. These methods use a supercritical fluid, such as carbon dioxide (CO_2), maintained under supercritical conditions (temperature and pressure). The SCF methods offers several advantages including a cheap and environmental harmless solvent, controlling of particle size, *in situ* sterilization and

possibility of large-scale production (Isalomboto Nkanga et al., 2019; William et al., 2020). The most used SCF methods are injection and decompression, rapid expansion of supercritical solutions (RESS), processes with supercritical CO_2 as an anti-solvent, gas anti-solvent (GAS), supercritical anti-solvent (SAS), aerosol solvent extraction systems (ASES) and supercritical reverse-phase evaporation (SCRPE) (Karn et al., 2013). Recently, other methods can be also employed to produce liposomes, such as dual asymmetric centrifugation and microfluidics (Huang et al., 2014; Meure et al., 2008). All the novel methods referenced above have an extremely potential future in the therapeutic and pharmacological applications (Karn and Hwang, 2015). The main characteristics, (+) advantages and (--) disadvantages of the novel methods are outline in Table 1, based in literature reviewing (Has and Sunthar, 2019; Huang et al., 2014; Karn et al., 2013; Maherani et al., 2011; Pattni et al., 2015).

Table 1. Main characteristics of the novel methods for liposome production.

Method	Main characteristics
Cross-flow injection (Wagner)	(+) Simple, scalable, continuous and sterile process. (--) Residual organic solvents can creates stability problems.
Membrane contractor	(+) Simple, rapid, scalable and continuous process; homogenous liposomes with higher EE for lipophilic drugs. (--) Less studied for hydrophilic drugs; high-cost material.
Cross-flow filtration	(+) Rapid, scalable, sterile process; homogeneous liposomes with high stability; facility to removal of detergent. (--) Understudy method.
Injection and decompression	(+) Sterile process; homogeneous liposomes by changing the nozzle diameter; narrow liposome size distribution; small organic solvent consumption.

	(--) Complex equipment with low yield; needs of high temperature and pressure; more adequate for hydrophobic drugs; nozzle can stay clogged.
RESS	(+) Simple fast and solvent-free process; liposomes with controllable size. (--) Low yield and EE.
GAS	(+) Suitable for a wide range of drugs; liposomes with variable size; and moderate stability; solvent-free and uncontaminated process. (--) Require organic solvent and needs gas and solvent separation; batch process.
SAS	(+) Simple, scalable; solvent-free and uncontaminated process; homogenous, small and stable liposomes; low use of organic solvent and moderate pressure and temperature. (--) Require organic solvents and needs gas and solvent separation; difficult to optimize conditions.
ASES	(+) Rapid, scalable and single step process; more adequate for dry liposomes; low organic residues. (--) Heterogeneous and large liposomes; uses a nozzle; understudy method.
SCRPE	(+) Simple, rapid and one-step process with scalable potential; no need for nozzles; reduced or no use of solvent; stable liposomes. (--) Understudy method; require high pressure; high-cost material.
Dual asymmetric centrifugation	(+) Simple, rapid and reproducible process; homogeneous and small liposomes; high EE for water soluble drugs. (--) Used only for small volumes; only laboratory-scale, not adequate for scale-up production, high pressure with agitation; understudy method.
Microfluidics	(+) Scalable process and used for biological samples; liposomes with controllable size. (--) Issues for thermolabile compounds; complex equipment; not adequate for scale-up production; difficult to clean after liposome production.

3.4. Drug loading methods

As mentioned before, liposomes are considered a good drug delivery system due their ability to load drugs with different characteristics (Laouini et al., 2012a). The selection of an adequate method for drug encapsulation into liposomes depends of several factors

such as EE, drug/lipid ratio, drug leakage and retention, sterility, facility of production and scale-up, cost efficiency and liposome stability (Maherani et al., 2011; Mayer et al., 1986). Furthermore, the amount of encapsulated drug is related with the kind of drug, the composition of liposomes and the method used for the liposomal production (Pattni et al., 2015). There are two different processes to encapsulate drugs into liposomes, namely passive and active methods (Akbarzadeh et al., 2013).

Passive loading method describes the procedure in which the drug is encapsulated during the liposome preparation. Hydrophilic drugs are dispersed in the aqueous phase (inside and outside of the liposomes), whereas hydrophobic drugs are located in the bilayer of the liposome (Maherani et al., 2011). In this procedure, immediately when they are being formed, liposomes can capture the aqueous volume containing the hydrophilic drug previous dissolved. Consequently, the concentration of the drug inside the aqueous core is similar to the aqueous volume enclosed by the liposomes. The EE of drugs encapsulated by passive loading changes due to numerous features, such as drug solubility, liposome size and charge, lipid concentration and production method (Pauli et al., 2019). The liposomal membrane is not permeable to ions and charged drugs. Otherwise, the uncharged drugs can diffuse through the lipid membrane, occurring drug leakage. Usually, this approach results in low EE, involving a large amount of non-encapsulated drug and high drug leakage for the drugs permeable to liposomal bilayer (Li et al., 2018). However, hydrophilic drugs that have protonizable amine functions can be encapsulated into liposomes by active loading, improving their EE in comparison with passive loading (Akbarzadeh et al., 2013).

The principle of active loading, also called remote loading, involves the creation of a transmembrane pH or ion gradient, that efficiently drives the drug through the lipid bilayer, leading to up to 100% loading in some drugs. This method is applied after the

liposome formation. The gradient is created between the inside of the intact liposomes (already formed) and the outside of liposome, the aqueous medium, where the drug is solubilized. As uncharged drugs can diffuse across the lipid membrane, they become protonated, inhibiting their diffusion out of the liposome, enhancing their EE and retention inside liposome (Li et al., 2018). The ideal loading efficiency is achieved when the drug is an amphipathic weak base (pKa ≤ 11) or weak acid (pKa > 3) (Zucker et al., 2009). There are several approaches to performed active loading, such as ammonium sulfate transmembrane gradient for amphipathic weak bases, calcium acetate gradient for weakly acidic drugs, phosphate gradient method, ethylenediaminetetraacetic acid (EDTA) gradient method and ionophore loading method (Li et al., 2018).

4. Characterization of liposomes

After production and before application, liposomes need to be extensively characterized for evaluation of their physico and chemical properties to guarantee their *in vitro* and *in vivo* performance (Çağdaş et al., 2014). The most investigated properties to characterize liposomes are size, size distribution (reported using the polydispersity index, PDI), surface charge (through zeta potential measurement), shape, lamellarity, phase behavior, EE, and *in vitro* drug release (Isalomboto Nkanga et al., 2019; Pattni et al., 2015). Table 2 summarizes the main analytical techniques used for the assessment of liposomal characteristics.

Table 2. Analytical techniques used for the evaluation of liposomal properties.

Properties	Analytical techniques
Size	Dynamic light scattering (DLS), Nanoparticle tracking analysis (NTA), Nuclear magnetic resonance (NMR), Field-flow fractionation (FFF), Size exclusion chromatography (SEC). Microscopy techniques: Transmission electron microscopy (TEM), Cryogenic-TEM (Cryo-TEM) and Atomic force microscopy (AFM).
Zeta potential	Laser Doppler electrophoresis (LDE) and Capillary electrophoresis.
Shape	Microscopy techniques: TEM, Cryo-TEM and AFM.
Lamellarity	Cryo-TEM, ^{31}P-NMR, Small-angle X-ray scattering (SAXS) and trapped volume determination techniques.
Phase behavior	Differential scanning calorimetry (DSC), Thermogravimetric analysis (TGA), fluorescence probe polarization, NMR, Electron paramagnetic resonance, Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD).
Encapsulation Efficiency	Ultraviolet-visible (UV–Vis) and Fluorescence spectroscopy, enzyme or Protein-based assays, High-performance liquid chromatography (HPLC), Ultra-performance liquid chromatography (UPLC), Liquid chromatography–mass spectrometry (LC–MS), Gas chromatography–mass spectrometry (GC-MS), Electron spin resonance (ESR) and ^{1}H NMR.
Drug release	Spectrophotometry methods, HPLC and UPLC.

4.1. Size and Polydispersity index

The size and PDI of liposomes are the most relevant features in liposome characterization. It has known that the liposome size shown to be a crucial factor for inhalation and parental administrations (Laouini et al., 2012b) and to determine the circulation half-life of liposomes (Elsana et al., 2019). While small liposomes can circulate in the organism for long time, large liposomes are more quickly eliminated from the blood circulation (Sercombe et al., 2015). For drug delivery, the desirable size of liposomes usually ranges

between 50 and 200 nm (William et al., 2020). The PDI value reveals in terms of size, the degree of sample heterogeneity, that can be monodisperse or polydisperse. PDI can be dimensionless and scaled such that values range from 0 to 1. In drug delivery applications using liposomes, a PDI value equal or below 0.3 indicates an acceptable and homogenous liposomal population (Danaei et al., 2018), whereas high PDI value is associated with a very broad size distribution (heterogeneity) or even several liposomal populations in the sample (Gaumet et al., 2008). The calculation of PDI is based on the particle size, refractive index of the solvent, the measurement angle and the variance of the distribution (Koppel, 1972).

The most used technique to measure these two features is DLS also known as photon correlation spectroscopy (PCS). DLS analyses the continuous motion of the dispersed particles in solution (Brownian motion), resulting in scattering of the incident light. The scattering of the light is correlated with the diffusion level of the liposomes in suspension (small particles diffused faster than the large particles). The evaluation of mean size is calculated based on the amount of light scattered. DLS is considered a simple, easy, fast and reliable method with the capacity to evaluate the liposome size in their native environment. Extensive range of measurement ability from a few nanometers to several micrometers is also applied (Isalomboto Nkanga et al., 2019; Pattni et al., 2015). However, this technique has some limitations, involving the difficult to differentiate single particles from aggregates and the high sensitivity to detect low amount of impurities (contaminants) (Fissan et al., 2014).

Recently, a size characterization tool called nanoparticle tracking analysis (NTA) was introduced to determine the size by measurement of the diffusion coefficient of particles in a sample (Kim et al., 2019). DLS determine the diffusion coefficient of particles based on the reads of the intensity change of scattered light. Whereas, NTA find the diffusion

coefficient by the movements of individual particles in successive optical video image. NTA can be a good approach to verify the size determined by DLS due they measure the same physical property. Therefore, the size measured by NTA should be similar to that observed in DLS technique (Filipe et al., 2010; Malloy and Carr, 2006). The capacity of NTA to simultaneously measure size and particle scattering intensity, besides allowing to distinguish particles of different refractive index within the same sample solution, makes a direct estimation of particle concentration (Elizondo et al., 2011).

4.2. Zeta potential

The overall net charge of the particles is usually expressed as surface or zeta potential (ζ-potential) (Kraft et al., 2014). This feature of liposomes is considered an essential physical property in the control of the electrostatic interactions between the particles in suspension (Kaszuba et al., 2010). The net charge of liposomes is influenced by key parameters, such as lipid composition, the head group of lipids and associated ligands, differing from negative, neutral, or positive. The ζ-potential can be also affected by the external environmental and its ionic strength (Manconi et al., 2003). The ζ-potential measurements are used to predict the stability of colloidal systems, such as liposomes in their surrounding medium. Usually, liposomes with low ζ-potential or uncharged have more probability to aggregate over time, because there will be no force to inhibit the liposomes flocculating. Otherwise, the liposomes in suspension with a large negative or positive ζ-potential charge present repulsive forces in the medium that prevents the natural tendency to aggregation (Laouini et al., 2012b).

The measure of ζ-potential needs a laser to provide a light source to illuminate the liposomes within the sample. The laser beam passes through the middle of the sample cell used to the measurements at a specific angle (Laouini et al., 2012b). Determination

of the surface charge permits the evaluation of fluctuations in the scattered light intensity caused by the particle motion in the suspension due to the application of an electric field. The charge of liposomes is proportional to their mobility rate (Hadinoto et al., 2013; Pattni et al., 2015). Posteriorly, the information is passed to a digital signal processor in a computer system and the value of ζ-potential is calculated by determining the electrophoretic mobility, i.e. a velocity of a particle in an electric field, and then applying one specific equation, called Henry equation (Laouini et al., 2012b; Prabhu and Murugan, 2015). LDE and capillary electrophoresis are the most known techniques used to measure the ζ-potential of liposomes through determination of their electrophoretic mobility (Monteiro et al., 2014b; Xiong et al., 2012).

4.3. Shape

The analysis of morphological characteristics, namely the shape, is vital for an adequate characterization of liposomes. The most select tool to ascertain the morphological features of liposomes is the microscopy (Elizondo et al., 2011). The visualization of liposomes as individual particles by microscopy techniques provides a direct observation of their shape. Electron microscopy techniques such as TEM and cryo–TEM have been widely implemented for creating liposomal images (Isalomboto Nkanga et al., 2019). TEM technique has some limitations at sample preparation level due the need to remove the native environment of liposomes. It is a time-consuming technique, thus is not flexible to being routine measurements. Moreover, this technique may induce alterations in liposomal shape, including possible vesicle shrinkage, swelling or artifacts formation in the created image (Chetanachan et al., 2008; Pattni et al., 2015). To overcome these limitations, another possibility is the use of cryo–TEM. This approach keeps the liposomes close to their native state and minimize the shape distortion or shrinkage by

involving the use of a flash freezing step with liquid nitrogen and then direct visualization of liposomes in a controlled environment. However, cryo–TEM usually works better with samples that have a lower nanometer range, because larger particles may be eliminated from the sample in the preparation step. The AFM technique appears for direct analysis of liposomes in their native environments without sample manipulation. It is considered a quick, powerful and non-invasive technique (Laouini et al., 2012b). The main advantage of this technique over electron microscopy is the high resolution of the micrographs at three-dimensional level with resolution down to the nanometer and Angstrom scales (Spyratou et al., 2009).

4.4. Lamellarity

Lamellarity is also a characteristic that can have an impact on the further liposomal applications due their influence on the EE and drug release profile. Cryo–TEM is the most used method and provide useful information regarding liposome lamellarity such as their bilayer thickness and inter-bilayer distance (Maherani et al., 2011). Other methods to access the lamellarity are based on the visible or fluorescence signal variations of lipids marker upon the addition of certain reagents (Isalomboto Nkanga et al., 2019; Laouini et al., 2012b). ^{31}P NMR approach has also been used to estimate the value of liposome lamellarity, particularly, the ratio of phospholipid amount in the outer to inner layers. The addiction of paramagnetic ions (Mn^{2+}, Co^{2+}, and Pr^{3+}) to the NMR sample preparation quenches the ^{31}P–NMR signal of the phospholipids. The interactions of the ions with the bilayer alter the NMR spectrum. Therefore, by comparison of both spectrum, before and after the incorporation of paramagnetic ions, it is possible to estimate the lamellarity (Fröhlich et al., 2001). SAXS and trapped volume determination are other techniques that

also be used to estimate the lamellarity of liposomes (Mayer et al., 1985; Pattni et al., 2015).

4.5. Phase behavior

As mentioned above, the Tc represents an important feature that can affect the fluidity of the lipid bilayer. For drug delivery applications, phase behavior is highly considered due the fact that the lipid bilayer permeability to entrapped hydrophilic drugs increases with lipid membrane fluidity (Craig et al., 1990). Several other liposomal properties including fusion, aggregation, stability and protein binding are also dependent on the phase behavior of a liposomal membrane (Maherani et al., 2011). Usually, the most common method used for study and determination of the Tc is the DSC. This thermal analysis technique is based on the evaluation of differences in heat flow, between a sample reference and a study sample. Both samples are subjected to a programmed heating, cooling or isothermal treatment using a meticulous control of the atmosphere, typically saturated with nitrogen gas (Isalomboto Nkanga et al., 2019). The Tc can be also measure by other methodologies such as TGA, fluorescence probe polarization, electron paramagnetic resonance, NMR, FTIR and XRD (Pentak, 2014; Sot et al., 2005). To calculate the phase behavior of phospholipids in lipid bilayers can be also explored the molecular dynamics simulations (Youssefian et al., 2017).

4.6. Encapsulation efficiency

An optimal exploration of liposome characteristics may permit to develop liposomal formulations with ideal EE and allow the control of drug release. The liposome composition, the method of liposome production as well as the rigidity of the bilayer membrane can have a crucial impact on the EE of a certain drug (Maherani et al., 2011).

Load the proper amount of drug to achieve the therapeutic efficacy is the key in the field of medical applications (Zucker et al., 2009). EE is calculated as the percentage of the amount of drug inside liposomes (encapsulated drug), compared with the total amount of drug used in liposomal preparation (encapsulated and non-encapsulated drug). The immediate result of liposome preparation contains a mixture of encapsulated and non-encapsulated drug fractions. Thus, the first step to quantify the amount of drug within liposomes and consequently determined the EE is the separation of the free drug (non-encapsulated). Numerous techniques have been used for this purpose, including size exclusion chromatography based on the differences in size (liposome versus free drug), gravitation or centrifugation, dialysis membrane with an appropriate cut-off and ultracentrifugation (Laouini et al., 2012b). The next step is the measurement of the amount of drug encapsulated into liposomes. There are two known ways to determine EE, namely the indirect and direct method. The indirect method focuses on assessing the non-encapsulated drug concentration in the eluted and subtract this concentration from the total drug concentration used in liposomal preparation. Otherwise, in the direct method the determination of EE can occur by direct disruption of liposomes with organic solvent and then the released material is quantified (Bakonyi et al., 2017). The conventional techniques used to estimate the concentration of drug encapsulated into liposomes depends mainly on their nature and include UV–Vis and fluorescence spectroscopy, enzyme or protein-based assays (Laouini et al., 2012b). Moreover, the determination of the amount of drug can be obtained using more sophisticate equipment such as HPLC, UPLC, liquid chromatography and gas chromatography mass spectrometry (LC-MS and GC-MS, respectively) (Edwards and Baeumner, 2006). Additional techniques such as ESR and ^{1}H NMR has also been used to quantify the amount of drug (Anzai et al., 1990; Zhang et al., 2004).

4.7. *In vitro* drug release

The evaluation of the *in vitro* drug release profile can be performed using dialysis conditions. The selection of dialysis bag membrane should be in accordance with the drug specifications. It must be freely permeable to the drug and should not occur drug adsorption (Laouini et al., 2012b). Liposomal sample is placed into the dialysis bag with specific molecular weight cut off, hermetically tied. The tubing membrane system is put into a simulated physiological fluid means release medium, usually a buffered saline at pH 7.4. The full system is kept at 37 °C to mimic an *in vivo* environment, and under continuous stirring. At defined time points, an aliquot of sample is taken and analyzed by the conventional methods used for drug quantification. The volume of samples needs to keep constant. Thus, an equal volume of fresh release medium is placed again in the system (Isalomboto Nkanga et al., 2019; Pattni et al., 2015). The data are used to establish the release profile by plotting the cumulative release percentage against the select time points. As extrapolation to *in vivo* performance of liposomes as drug delivery system, the results obtained from the *in vitro* release study are widely considered in the development of liposomes for the controlled release of drugs (Dash et al., 2010).

5. Classification of liposomes

Liposomes compared to others colloidal delivery systems offers the advantage to alter their structural and physicochemical characteristics. Therefore, it is possible to modify liposomes behavior *in vivo* and targeting liposomes to a specific site in the organism. Liposomes can be classified based on their composition and functionalization. In addition to conventional, stealth and targeted liposomes, the recent improvement on the design of liposomes leads to a different types of liposomes such as immunoliposomes and stimuli

responsive liposomes (Allen and Cullis, 2013; Nisini et al., 2018). The differences between these categories of liposomes will be highlighted below.

5.1. Liposome composition and functionalization

Since their discovered, liposomes have been produced with different characteristics based on their composition and functionalization (Fig. 3). The first generation of liposomes to be used in therapeutic applications was the conventional liposomes (Abra et al., 2002; Cattel et al., 2004; Immordino et al., 2006). These liposomes can be composed of neutral, cationic or anionic charged phospholipids, usually in combination with CH to promote the stabilization of the liposomal bilayer (Monteiro et al., 2014a; Sercombe et al., 2015), as previous explained. However, this type of liposomes continues to be subjected to several difficulties, such as the instability in plasma which results in short blood circulation half-life. Liposomes are rapidly captured by RES and removed from the blood circulation (Immordino et al., 2006). The binding of opsonins, serum proteins, to the liposomes is the first signal for liposomes elimination. Opsonins, recognize the conventional liposomes as foreign particles, and consequently they are destroyed by phagocytes of the MPS (Riaz et al., 2018).

To overcome the difficulties of conventional liposomes, a second generation of liposomes was developed, led to the creation of so-called stealth, long-circulating or PEGylated liposomes (Saraf et al., 2020). The stealth strategy involves mainly the possibility to coat the liposomal membrane surface with biocompatible hydrophilic polymer conjugates, such as PEG, chitosan, and others, increasing repulsive forces between liposomes and serum-components (Hatakeyama et al., 2013). Therefore, results the reduction of immunogenicity and macrophage uptake, enhancing its blood circulation half-life and reducing the toxicity of encapsulated compound (Madni et al., 2014). The methods to

anchor the PEG in the liposome membrane involves the physical adsorbing of the polymer onto the surface of the liposomes, the incorporation the PEG-lipid conjugate during liposome preparation, or thought the covalent attachment of reactive groups onto the surface of preformed liposomes (Immordino et al., 2006). However, an important restriction of stealth liposomes is their large body biodistribution. Thus, the encapsulated compound cannot be selectively delivered to specific target cells (Torchilin, 2005). From this limitation, ligand-targeted liposomes were designed for targeted delivery of compounds at the desired tissues, promoting higher and more selective therapeutic activity (Immordino et al., 2006). In addition to surface modification of liposomes with PEG, targeted liposomes are also functionalized using glycoproteins, polysaccharides, or a ligand for specific receptors, such as antibodies, small molecules or peptides (Riaz et al., 2018; Torchilin, 2005). The ligand can target specific receptors which are overexpressed on the surfaces of the diseased cells, binding to them, resulting in a minimum off-target effects to healthy cells (Fathi and Oyelere, 2016; Le et al., 2019).

Following the principles of the previous strategy, it was considered the design of antibody-functionalized liposomes (immunoliposomes) and stimuli-responsive liposomes (Nisini et al., 2018). Immunoliposomes are formulated by chemically coupling of antibodies or their fragments to the liposomal surface, resulting in target antigens with an elevated degree of specificity (Eloy et al., 2017). In a stimuli sensitive liposomal system, the release of the drug occurs upon changes in some physicochemical or biochemical stimuli, such as pH, temperature, redox potential, enzymes and electrolyte concentrations, ultrasound, electric or magnetic fields (Drummond et al., 2000; Karanth and Murthy, 2007). The most common examples of stimuli-responsive liposomes are the pH-sensitive and temperature-sensitive liposomes (Li et al., 2010; Lu et al., 2014). Additionally to the delivery of drugs, liposomes can be used for other purposes, with

simple modifications on their composition and charge (Nisini et al., 2018). A good example is the use of cationic liposomes in gene therapy as transfection vectors, to the delivery of genes. The encapsulation of genes into liposomes, permits the protection of nucleic acids against degradation during storage and in the systemic circulation (Immordino et al., 2006).

More recently, multifunctional liposomes have been studied for their potential to perform a combination of multiple functions through surface modification techniques, resulting in liposomes with a wide range of functionalities (Riaz et al., 2018). In literature have been reported several examples of multifunctional liposomes. One example is the theranostic liposomes, at the same liposome it is possible to have an imaging and therapeutic agent (diagnosis and treatment functions) (Li et al., 2012; Sercombe et al., 2015). Another example is the dual-targeting liposomes that involves liposomes having two different ligands (Riaz et al., 2018).

Fig. 3. Different types of liposomes used in therapeutic applications.

5.2. Targeting strategies of liposomes

Almost as intense, an area of research and development of the liposome formulation is their targeting strategies. The specific targeting is a primordial functional property of liposomes as drug delivery systems (Zylberberg and Matosevic, 2016). Thus, targeting of specific sites focuses on both the development of new diagnostic tools and improving the efficacies of therapeutic agents (Fay and Scott, 2011). Currently, there are two main strategies by which targeting of liposomes can be broadly classified, namely passive and active targeting. Passive tissue targeting is mainly achieved through properties of cancer

vasculature, and active tissue targeting through receptor-specific ligands on the liposome surface intended for cell binding (Fig. 4) (Lehner et al., 2013).

Fig. 4. Schematic illustration of passive and active targeting strategies of liposomes into a tumor for enhancing the therapeutic efficacy of drugs.

5.2.1. Passive targeting

Passive targeting approaches have been mainly applied in the oncology field due to pathophysiological features of cancers and their environment (Wicki et al., 2015). Passive targeting of liposomes to tissues or cells is performed by transport and delivery them into the tumor interstitium via leaky tumor vasculature through molecular drive within fluids (Gogoi et al., 2016). In this way, non-targeted liposomes ranging from 10 to 500 nm in size can accumulate preferentially on the tumor and inflamed tissues via the enhanced permeability and retention (EPR) effect of the vasculature, because of abnormal leaky blood vessels and lack of functional lymphatics (Biswas and Torchilin, 2014; Fang et al., 2011; Torchilin, 2011). Passive targeting involves the needs to develop a liposomal formulation that can avoid their rapid elimination by organism defense mechanisms, such as phagocytic uptake or clearance by the cells of the MPS (Kraft et al., 2014). Thus, the preparation of stealth liposomes can be a good example to be used in passive targeting approaches due to surface modification of liposomes with PEG, that permits increase their circulation time (Zylberberg and Matosevic, 2016). This strategy, also involves the use of typical features of liposomes, such as their charge, that can induce the specific targeting to the cancer cells. Another example, can be the cationic liposomes. This type of liposomes is found to bind the negatively charged phospholipid head groups, specially expressed on tumor endothelial cells by electrostatic interactions (Byrne et al., 2008). The

mechanism of targeting based only on the EPR effect is not enough to complete attenuate the side effects of cytotoxic drugs. The heterogeneity of EPR effect within tumors and their limitation to some solid tumors, can also affect the efficacy of drugs delivered by passive targeting (Kraft et al., 2014; Park et al., 2019). Therefore, the development and searching of alternative targeting approaches with advanced functionalities such as active targeting have been explored (Wicki et al., 2015).

5.2.2. Active targeting

In 1906, the visionary Paul Ehrlich introduced the concept of active targeting by describing a "magic bullet" needed to direct specific drug delivery within the body (Lehner et al., 2013; Strebhardt and Ullrich, 2008). Since then, researchers worldwide have been searching for the "magic bullet" that would target selected cells with precision facilitating diagnosis and therapy (Bazak et al., 2015).

Active targeting involves the attachment of a targeting ligand to the surface of liposomes for enhanced delivery of liposomal systems (Riaz et al., 2018). Numerous targeting ligands have been employed to active targeting, including antibodies, nucleic acids (aptamers), peptides and whole proteins (*e.g.*, transferrin) and small molecules such as vitamins (*e.g.*, folic acid) (Wicki et al., 2015). There are several aspects considered in the selection of target ligands, which include: relative degree of over-expression or selective expression on the target, target cell uptake of the ligand-targeted formulation, and degree of covering of the target molecule (Noble et al., 2014; Sawant and Torchilin, 2012). These ligands should also be selected to allow binding to the target cells while minimizing binding to healthy cells (Lehner et al., 2013; Torchilin, 2005).

There are three main approaches available to functionalize liposomes. The first is binding the desired targeting ligand to a lipid prior to mixing them with other lipid components

during liposome preparation. In second approach, immediately after preparation, liposomes are functionalized with the required targeting ligand (Marqués-Gallego and De Kroon, 2014). Head group modified lipids with a PEG spacer functionalized at the end with amine, carboxylic acid, thiol or maleimide groups represent available options for this approach (Conde et al., 2014). In another methodology, it was proposed the post-insertion of the functionalized lipid in preformed liposomes. This method is based on the spontaneous incorporation of functionalized lipids from the micellar phase into preformed and even drug-loaded liposomes. Derivatization of the targeting molecule happens in a separated step, as an approach to prevent the interference of activated lipids with other liposomal components such as those present in the buffer (Steenpaß et al., 2006).

6. Limitations of liposomes

The stability of liposomes is a key consideration in drug delivery applications. Indeed, the therapeutic effect and safety of liposomes encapsulating drugs depend on their lifetime and their distribution within the body, and these features are directly related with their stability (Taira et al., 2004). The stability is considered the main concern for liposome preparation, storage and further administrations steps (Laouini et al., 2012b). The potential instability issues of liposomes are typically related to oxidation and/or hydrolysis of lipids, drug leakage, aggregates formation or even liposomal fusion (Immordino et al., 2006). Another challenge of liposomal formulations is the identification of a suitable large-scale production method and the needs to found an efficient sterilization technique for liposomes. These limitations of liposomal formulations will be addressed below.

6.1. Liposome stability

Liposomes itself are considered a moderately unstable colloidal system. A stable liposomal form preserves its physical integrity and does not negatively stimulus the chemical integrity of the encapsulated drug during its life (Laouini et al., 2012b). The evaluation of liposomal stability includes the verification of some specific parameters such as (i) the chemical and physical stability, (ii) the conservation of their size and structure, (iii) the maintenance of encapsulated drug and (iv) the impact of biological fluids on the liposomal properties (Monteiro et al., 2014a). Thus, these parameters can be interrelated. According to Food and Drug Administration (FDA), liposomes needs to be stable at least two years to be considered a liposomal drug product (Maherani et al., 2011). Chemical and physical stability are the main critical issues that influence the final performance of liposomes at biological level (Antimisiaris et al., 2007). Normally, the evaluation of size and the visual inspection of liposomes appearance are two principal features to determine the liposomal physical stability. This event is related to the tendency to agglomeration or aggregation. Thus, fusion and breakage of liposomes on storage can also leads to drug leakage from liposomes (Laouini et al., 2012b). Chemical stability can be considered the aptitude of liposomes to preserve the level of EE when changes in the medium can be occur, including pH alterations, electrolyte composition, oxidizing agents, and presence of surface active compounds (Maherani et al., 2011). The most important component in liposomes is the lipid. In its turn, lipids contain unsaturated fatty acids and can suffer oxidative reactions that can be stimulated by light, metal ions or temperature (Monteiro et al., 2014a). Chemical degradation may induce permeability changes within lipid membrane. Additionally, the interactions between the drugs and phospholipids can also interfere in liposomal chemical stability. The control of microbial stability of liposomal formulations is also important due the therapeutic formulations of liposomes

are parenteral products and must be sterilized to remove the microbial contaminants from the final product (Laouini et al., 2012b).

6.1.1. Freeze-drying

Liposomal formulations can be stored in an aqueous solution or in a dry powder form (Sebaaly et al., 2016). To overcome the main instability issues of liposomes in an aqueous solution, their storage in a dry state can be an attractive way for long-term stability (Chen et al., 2010). Among the feasible methodologies, freeze-drying, also known as lyophilization, remains the most studied and applied technique for this purpose. Freeze-drying consists on water removal from a frozen sample by sublimation and desorption under vacuum. However, the complexity of the process itself can compromise the liposomal membrane integrity from stresses caused by the freezing and drying steps. Thus, the choice of an ideal conditions to lyophilization is the main challenge to origin a final product with adequate characteristics, such as (i) elegant cake appearance with fast reconstitution time, (ii) suitable physico-chemical characteristics after reconstitution, (iii) low water content and (iv) satisfactory long-term stability of final liposomal formulation (Abdelwahed et al., 2006). The use of an appropriate excipient within the liposomal formulation, can maintain their size and avoid their drug leakage (Janicki et al., 2002). The excipients are included to protect the liposomes in the main steps of the freeze-drying process, cryoprotectants assist in freezing stress and lyoprotectants contribute in drying stress. Table 3 represents the most used excipients in freeze-drying of pharmaceutical products (Abdelwahed et al., 2006).

Table 3. Examples of commonly used excipients in freeze-drying of pharmaceutical products.

Type of excipient	Main characteristics	Excipient
Bulking agents	Offers bulk to the formulations, in the case of very low concentration of the product to be freeze.	Trehalose, mannitol, lactose, hydroxyethyl starch and glycine.
Buffers	Regulate pH changes during freezing.	Phosphate, tris hydrochloride, citrate and histidine.
Stabilizers	Protect the liposomes during the lyophilization process, including freezing drying stresses.	Sucrose, lactose, glucose, trehalose, glycerol, mannitol, sorbitol, glycine, alanine, lysine, PEG and dextran.
Tonicity adjusters	Control the osmotic pressure and produce an isotonic solution	Sucrose, mannitol, glycine, glycerol and sodium chloride.
Collapse temperature modifiers	Obtain higher drying temperatures increasing the collapse temperature.	Hydroxypropyl-β-cyclodextrin, PEG and dextran.

The formulation features, including the liposomal composition, the nature of the drug as well as the type of excipient are the main responsible by the protective effect during lyophilization. Therefore, an exhaustive optimization of these features can be an appropriate way to improve the stability of the liposomes after lyophilization. The most used excipients are the sugars such as trehalose, sucrose and glucose. The sugars are considered ideal stabilizers to protect liposomal integrity during the lyophilization process. The stabilizer effect promoted by sugars depends on their nature and concentration. Thus, these parameters must be careful selected and optimized to guarantee an enhanced stabilizer effect of lyophilized liposomes (Fig. 5) (Abdelwahed et al., 2006).

Fig. 5. Effect of cryo/lyoprotection on the size of liposomes after the freeze-drying process.

6.2. Scale-up and sterilization methods

The major limitation of liposomes application is the identification of a suitable method for large scale production as known as scale-up. To use liposomes as an acceptable pharmaceutical product, their production at large scale needs to be easily and economically feasible (Laouini et al., 2012b). The slowed develop in scale-up process is associated to the time dispensed to resolve problems involving the quality and technological control. These problems included (i) accessibility of high-quality lipid raw materials, (ii) validated quality control analyses, (iii) unavailability of equipment, (iv) reliability and reproducibility batch to batch, (v) efficient and valid sterilization methods and (vi) long-term stability of produced liposomes. All these problems can be interrelated (Barenholz and Lasic, 2018; Saraf et al., 2020). As discussed in section 3, there are several methods available for production of liposomes at laboratory scale. However, only a few manufacturing techniques are available at industrial scale (Wagner and Vorauer-Uhl, 2011). The production of liposomes involves an amount of unit operations which are not easy to transpose for commercial manufacturing (Toh and Chiu, 2013). The key issue for production of a successful liposomal formulation at industrial scale is the control and keep constant the characteristics of each batch maintaining the reproducibility of the method (Langer et al., 2008). At laboratory scale, usually is easy to reach the reproducibility of the process, whereas at industrial scale the PDI of liposomes is difficult to control and the reproducibility of batch-to-batch is challenging to achieve (Zamboni et al., 2012). The ethanol injection method is considered the most interesting technique for this purpose due the reproducibility and fast implementation of this method (Charcosset et al., 2015; Justo and Moraes, 2011).

Another limitation of liposomes is their sterilization that remains a challenging issue due the susceptibility of liposomes to physical and chemical degradation. Methods for

liposomes sterilization should be a compromise between the inactivation of the microorganism's contamination and the degradation of liposomal product. The sterilization methods should not affect the physical and chemical characteristics of liposomal formulation and should be destructive for the microorganisms (Barenholz and Lasic, 2018). The most common technique to achieve sterilize small liposomes is the filtration using a sterile polycarbonate membrane with adequate pore size, normally 0.22 μm, under aseptic conditions (Laouini et al., 2012b). This method has the advantage that is not destructive for small liposomes. Filtration is not appropriated for liposomes with high values of size (> 0.22 μm) and for large volume of liposomes due the possibility of filter clogging which compromises the final product. It should be noted that there are other methods for liposomes sterilization, for example, autoclaving, high pressure sterilization using nitrogen gas, utilization of saturated steam to sterilize pharmaceutical equipment, ethylene oxide treatment, UV sterilization, γ-irradiation and dense gas technique (Barenholz and Lasic, 2018; Toh and Chiu, 2013). However, it is important to note that conditions required in these conventional sterilization techniques can be detrimental to the stability of the liposomal preparations (Toh and Chiu, 2013).

7. Therapeutic applications of liposomes

Liposomes have been revealing promising results as drug delivery system for numerous kinds of drugs. Thus, the intensive investigation of liposomes in medicine led the researches to develop different liposomal formulations for the controlling and management of a wide range of diseases besides an extensive variety of therapeutic applications. The encapsulation of drugs inside liposomes improve their therapeutic effect due the pharmacokinetics and pharmacodynamics alterations (Bulbake et al., 2017). The modulation of the *in vivo* drug behavior and the reduction of the drug toxicity in the

organism are the crucial features to design a suitable liposomal formulation. The use of liposomes in clinical applications focuses in the treatment and diagnosis of cancer. However, the potential of liposomes for therapeutic applications is not limited to cancer therapy. Liposomes are considered an extremely flexible platform and can be used in diverse field of research (Maurer et al., 2001). In this section will be explored the current market liposomes and the use of liposomes specifically in rheumatoid arthritis therapy.

7.1. Marketed liposomes

Liposomes have revealed significant therapeutic benefits in clinical applications. However, their applicability is limited due to the all stages of liposomal development and production process that comprises manufacturing methods, regulatory approval by the competent authorities and intellectual property (Saraf et al., 2020). Despite all the intensive research in the development of liposomal formulations to use in therapeutic application, in the moment, only a few liposomes have entered in the market as a commercialized liposomal product (Moosavian et al., 2019).

The first successful liposomal formulation, Doxil®, was introduced to the USA market in 1995 and it is the first liposomal product to obtain regulatory approval by FDA. Doxil®, or Caelyx® in Europe, is an intravenous injection product that contain doxorubicin (DOX) hydrochloride in their formulation. Doxil® is used to treat advanced ovarian cancer and acquired immunodeficiency syndrome (AIDS)-associated Kaposi's sarcoma, after the inefficiency of prior chemotherapy or intolerance therapies (Bulbake et al., 2017). These liposomes proved to improve the pharmacokinetic properties of free DOX and minimize the life-threatening toxicities caused by the drug. Despite cancer therapy is the most studied area in terms of liposomal clinically approved products, liposomal products were also investigated for other diseases. Fig. 6 identifies the main

therapeutic fields covered by liposomal formulations products (Bulbake et al., 2017). The product name, active agent and pharmacological indications are also referenced. It can be prepared in different forms, liquid (suspension), solid (dry power) and semi-solid (gel or cream). The administration of liposomes in *vivo* can be topically or via parenteral route (Laouini et al., 2012b).

Fig. 6. Main therapeutic fields covered by liposomal formulations products (adapted from (Bulbake et al., 2017)).

It is important to highlight that most of liposomal products developed are nowadays under different pre-clinical studies and clinical trials. The translation of liposomes for clinical trials requires advanced models and methodologies. These models can predict the biosafety of liposomes inside the organism to enhanced their therapeutic applications (Saraf et al., 2020).

8. Conclusion

Liposomes have gained extensive attention as drug delivery system for numerous kinds of drugs. The direct application of liposomes in medicine encourages the researchers to create novel liposomes for treatments and diagnosis in a wide range of diseases as well as in a variety of therapeutic applications. In the context of liposomal therapy, the modulation of the *in vivo* drug behavior and the reduction of the drug toxicity in the organism are the crucial features to design a proper liposomal formulation. A suitable liposomal formulation product consists in three essential components, lipids to form a liposome, molecules to functionalized them and a drug molecule that will be encapsulated. As we can see from this review, the development and improvement of

liposomes are a complex challenge that involves the simultaneous optimization of several parameters to achieve a final liposomal formulation safe and effective. Although there are actually some liposomes approved on the market covering many health areas, it is possible to claim that there is still much to be done in the field of liposomal technology to overcome the limitations explored in this review. In summary, liposomes can contribute to treatments with key performance, hence it shall lead to a better clinical outcome, lower toxicity levels and fewer side effects.

Declaration of interest

The authors report no conflicts of interest.

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Design of liposomes as drug delivery system for therapeutic applications

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Highlights

- Liposomes represent a powerful delivery system for several drugs;
- Methods for liposomal production: conventional and novel;
- Properties of the liposomal formulation such as its characterization, stabilization and limitations.
- Therapeutic applications of liposomes, focuses on marketed liposomes.

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Eugénia Nogueira: Supervision, Conceptualization, Writing - Review & Editing.

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Conflict of Interest

The authors report no conflicts of interest.