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# General Perception of Liposomes: Formation, Manufacturing and Applications

*Christian Isalomboto Nkanga, Alain Murhimalika Bapolisi, Nnamdi Ikemefuna Okafor and Rui Werner Maçedo Krause*

## Abstract

Liposomes are currently part of the most reputed carriers for various molecular species, from small and simple to large and complex molecules. Since their discovery, liposomes have been subject to extensive evolution, in terms of composition, manufacturing and applications, which led to several openings in both basic and applied life sciences. However, most of the advances in liposome research have been more devoted to launching new developments than improving the existing technology for potential implementation. For instance, the evolution of the conventional lipid hydration methods to novel microfluidic technologies has permitted upscale production, but with increase in manufacturing cost and persistent use of organic solvents. This chapter intends to present general concepts in liposome technology, highlighting some long-standing bottlenecks that remain challenging to the preparation, characterization and applications of liposomal systems. This would enhance the understanding of the gaps in the field and, hence, provide directions for future research and developments.

**Keywords:** phospholipids, soybean lecithin, liposome composition, manufacturing methods, characterization techniques

## 1. Introduction

Liposomes are artificial lipid-based bilayered vesicles. They were firstly discovered and described in 1965 by Bangham et al. [1] as swollen phospholipid systems, namely Banghasomes. A few years later, the structural description of liposomes was unveiled as small devices made of one or more closed phospholipid bilayers. Due to the diversity of particle sizes, from 20 nanometers to several micrometers, liposomal vesicles are considered as either nanoparticles or microparticles endowed with the ability to encapsulate materials of various nature and polarity [2, 3].

Up until now, liposomes have shown huge promise as potential vehicles for biologically active compounds in cosmetic and pharmaceutical industries. These applications have been extended to food and farm industries, where unstable substances such as antioxidants, flavors and antimicrobials have been explored for liposomal encapsulation. Across all these areas of application, liposomes have been deemed to be the most successful delivery systems due to their multiple advantages. These include high biocompatibility and biodegradability, low toxicity and poor immunogenicity, improved drug solubility and controlled distribution, as well as

the ability of performing surface modifications for targeted, extended and sustained release. Currently, there are several liposomal formulations that are clinically established for the treatment of various diseases, such as cancer, fungal and viral infections; and many more have reached advanced phases of clinical trials [4, 5].

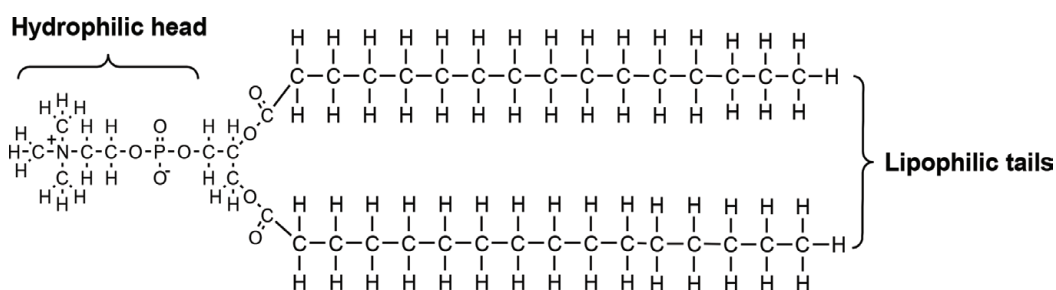
Although liposomes have shown some success in drug product development, the limitations identified in liposomal technology have remained almost stagnant over decades. The most common disadvantages of liposomes arise partly from poor stability under shelf and in vivo conditions. This is mostly due to potential lipids oxidation and hydrolysis, leakage and loss of hydrophilic cargoes, as well as particles fission and fusion. To date, some of these problems can be circumvented by playing around formulation adjuvants, such as anti-oxidants, or post-preparation processing, such as freeze-drying [4, 6, 7].

While describing broadly the current perception of liposomes, regarding production, evaluation and applications; this chapter intends to highlight the longstanding bottlenecks that remained overlooked and challenging to product development and implementation. This would increase the understanding of the gaps in the field and provide future directions to new openings for improvements in liposome technology.

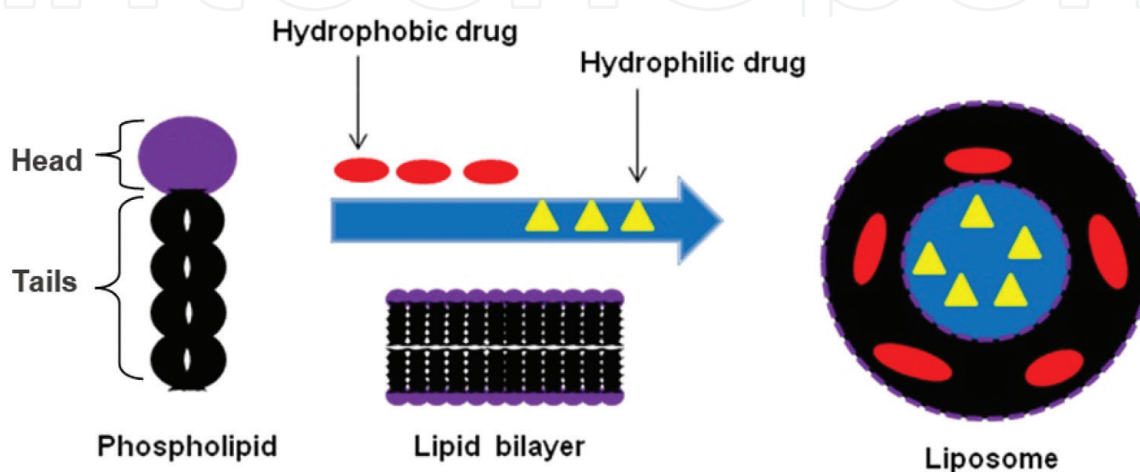
## 2. Liposomes formation and classification

### 2.1 Liposomes formation

The liposomal vesicles derive from hydration of phospholipids, which are amphiphilic molecules endowed with a hydrophilic head group and two hydrophobic acyl chains (**Figure 1**). In aqueous media, phospholipid molecules self-assemble



**Figure 1.**  
Chemical structure of a representative phospholipid molecule (distearoyl phosphatidylcholine).



**Figure 2.**  
Flowchart illustrating liposome formation and encapsulation of drug molecules.

Vesicle designation	Main components	Illustrative application
Emulsomes	A mixture of fats and triglycerides stabilized by high proportion of lecithin	Emulsomes loaded with Amphotericin B for the treatment of visceral leishmaniasis [9]
Enzymosomes	Complexes of lipids and enzymatic proteins	Encapsulation and delivery of superoxide dismutase for oxidative stress management [9]
Sphingosomes	Sphingolipids containing amide and ether bonds	Sphingosomes loaded with vincristine (Marqibo <sup>®</sup> ) for lymphoblastic leukemia therapy [4]
Transfersomes	A mixture of single chain surfactant, phospholipids and ethanol (10%)	Transfersomes loaded with diclofenac for improved topical delivery/retention [10]
Ethosomes	Phospholipids and ethanol (20–40%)	Mitoxantrone-loaded ethosomes for the treatment of melanoma [11]
Pharmacosomes	Conjugate of drug and phospholipid	Pharmacosomes loaded with diclofenac for enhanced the bioavailability and reduced toxicity [9]
Virosomes	Viral glycoproteins	Virosome containing HIV-1 gp41-subunit antigens for protection against vaginal simian-HIV [12]
Aquasomes	Tin oxide, diamonds or brushite core covered with oligomeric film	PEG-lipid coated aquasomes containing interferon- $\alpha$ -2b for prolonged and enhanced cytotoxicity [13]
Bilosomes	Bile salts and acids (deoxycholic acid)	Bilosomes loaded with diphtheria toxoid for systemic and mucosal immunization [14]
Niosomes	Non-ionic surfactants (span and tween)	Niosomes based formulation for enhanced oral bioavailability of candesartan cilexetil [15]

**Table 1.**  
 Presentation of liposome-type systems.

into a bilayered structure. Within the bilayer, phospholipid polar groups line up to form a water-attracting surface while their lipophilic chains face each other to yield a water-free zone. On mechanical shaking or heating, phospholipid bilayers continuously enclose the dispersing aqueous medium and form a vesicular system. In this system, hydrophilic groups of phospholipids are oriented towards the inner and outer aqueous phase, while their hydrophobic tails are centered within the bilayer [2, 4]. This architecture underlines the ability of liposomes to readily encapsulate hydrophilic and hydrophobic materials inside the inner aqueous core and the lipid bilayers, respectively (as illustrated in **Figure 2**).

## 2.2 Liposomes classification

Depending on the particle size and number of bilayers forming the vesicles (lamellarity), liposomes can be categorized in the following classes [4, 8]:

- Small unilamellar vesicles (SUV), size range 20–100 nm;
- Large unilamellar vesicles (LUV), size >100 nm;
- Giant unilamellar vesicles (GULV), size >1000 nm;
- Oligolamellar vesicles (OLV), size range 100–1000 nm;

- Multilamellar large vesicles (MLV), with size >500 nm;
- Multivesicular vesicles, size from 1000 nm to several thousand nanometers.

Based on their composition, liposomes can be classified as conventional, long circulating, cationic, stimuli-responsive and immunoliposomes. The differences between these categories will be highlighted later when discussing composition and evolution of liposomes.

Furthermore, there are many other vesicular systems considered as part of the liposome-type vesicles. These include emulsomes, enzymosomes, sphingosomes, transfersomes, ethosomes, pharmacosomes and virosomes, which are lipid-based liposomes analogous. The non-lipid-based liposomes analogous are aquasomes, bilosomes and niosomes [8, 9]. All the liposome-type systems are briefly presented in **Table 1**.

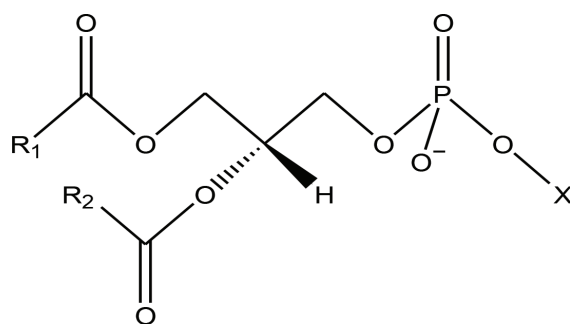
### 3. Liposomes composition and evolution

#### 3.1 Liposomes composition

Liposomes are made of physiologically acceptable natural or synthetic phospholipids found in the lipid bilayer membranes of human cells. The most frequently used phospholipids for liposomes preparation are phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS) and phosphatidylglycerols (PG) [16]. The molecular structures of these biocompatible lipids are shown in **Figure 3** and **Table 2**.

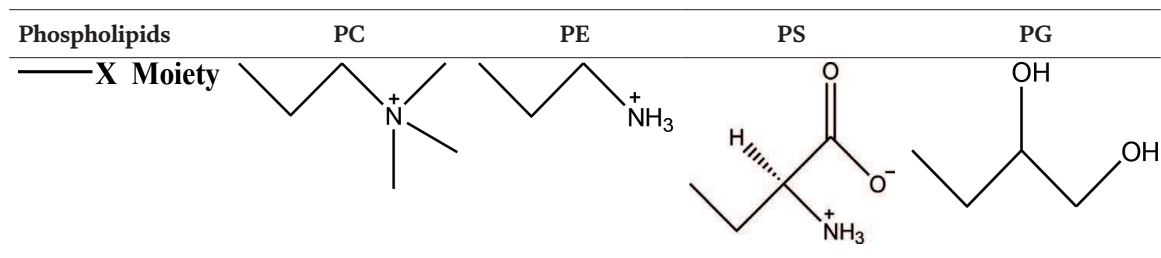
In liposomal technology, a considerable attention is given to the phase transition temperature (T<sub>t</sub>) of these phospholipids. The T<sub>t</sub> corresponds to the temperature above which phospholipids exist in liquid crystalline phase. In this fluid state, hydrophobic tails of phospholipids are randomly oriented but ready to form closely continuous bilayered vesicles (liposomes). Below the T<sub>t</sub>, phospholipids exist in gel state, where the hydrophobic tails are completely expanded and well packed, thus not able to form liposomes [4, 17].

As most of the phospholipids used for liposomes formulation have T<sub>t</sub> close to the physiological temperature (37°C), the addition of cholesterol has been adopted as a strategy to stabilize the liposomal vesicles in physiological media. This is especially for phospholipids that can undergo phase transition and leakage at room temperature, which can lead to premature release of the liposome cargo. In fact, due to its high hydrophobicity, cholesterol was found efficient in strengthening the packing of phospholipid bilayers, reducing therefore membrane permeability. Numerous



**Figure 3.** Structural representation of ester glycerol-phospholipid molecules, with R<sub>1</sub> and R<sub>2</sub> representing the hydrocarbon chains of different fatty acids.





**Table 2.**  
 Description of  $-X$  moieties of different glycerol-phospholipids [16].

studies have reported the ability of cholesterol to impact liposomes properties and functionality, including encapsulation efficiency and release characteristics [18–20]. The work by Kirby et al. [21] demonstrated that increasing cholesterol content can prevent leakage and improve in vivo stability of liposome. Later, Lopez-Pinto et al. [22] observed a direct correlation between cholesterol content and liposome sizes. These observations have established cholesterol content to be a key parameter in liposome formulation.

Like cholesterol, there are many other ingredients that can affect liposomes behavior and afford the desired encapsulation or delivery profiles. Additive agents such as oleic acid and N-[1(2,3-dioleoyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTAP) are useful for the preparation of negatively and positively charged liposomes, respectively. These charged liposomes offer the advantage of great liposomal stability during the storage, as charged particles repel each and reduce aggregation tendencies. While the cell internalization of positively charged liposomes (cationic liposomes) is promoted by their electrostatic interaction with cell membranes (which are negatively charged), liposomes bearing negative charges are subjective to poor cell internalization due to the corresponding repulsive forces. Cationic liposomes are used in gene therapy due to their ability to successfully encapsulate nucleic acids by electrostatic forces [4, 23].

In addition, some special lipids such as cholesteryl hemisuccinate (CHEMS) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), have been widely used to prepare liposomes with pH-dependent release features. CHEMS can exhibit pH-sensitivity either alone or in the presence of other lipids. In ionized forms at basic or neutral pH, CHEMS stabilizes the lamellar form of DOPE in lipid-based vesicles. However, the protonated or molecular CHEMS formed at acidic pH promotes hexagonal phase of this lipid, which leads to the disruption of the vesicular systems and release of the encapsulated materials [24, 25]. Tocopherol hemisuccinate (THS) has also shown similar pH-responsiveness as CHEMS, due to their molecular similarity [26].

The composition of liposome appears to be a broad topic, but also very crucial for the desired product development. However, the nature and costs of the liposome components used over decades viz., particularly the synthetic or highly purified natural phospholipids, have been reported to be part of the factors affecting negatively the universal implementation and affordability of liposome technology [16, 27]. The review by Machado et al. [28] discussed the feasibility of using crude soybean and rice lecithin for liposomal encapsulation of food ingredients. The authors demonstrated these naturally occurring phospholipid mixtures could be useful for liposomes preparation regardless of the intended area of application. Our group has recently investigated liposomal encapsulation of isoniazid using crude soybean lecithin. The formulated liposomes exhibited much better encapsulation efficiency than purified soybean lecithin [29]. This study proposed crude soybean lecithin for liposomal encapsulation of drug molecules. However, the complexity of this lipid mixture might be a bottleneck for some biomedical applications, where molecular architecture of the lipid bilayer is explored to get insights into potential

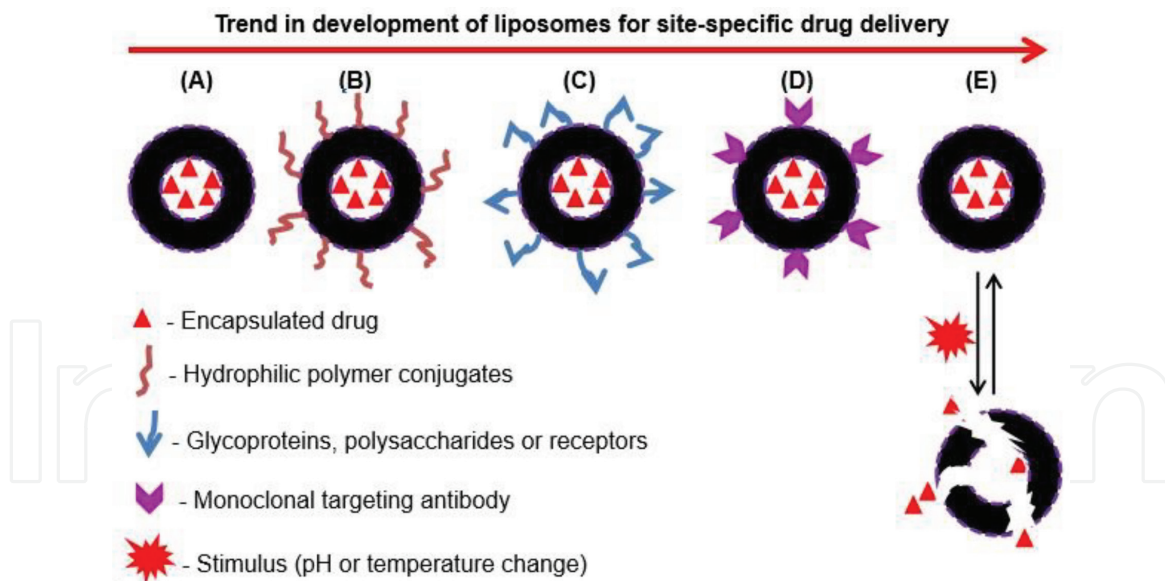
cell membrane permeability. The versatility of crude soybean lecithin liposomes is therefore in question, considering the wide range of areas that the liposomal systems usually cover.

### **3.2 Liposomes evolution**

Based on the composition, liposomal systems can be considered to have evolved from conventional, long circulating, targeted and immune-liposomes to stimuli-responsive liposomes. The liposomes composed purely of phospholipids with or without cholesterol (conventional liposomes) have shown some limitations due to their uptake by the cells of the mononuclear phagocytic system (MNPS), such as macrophages that ensure liposomes clearance through phagocytosis. This biological fate makes conventional liposomes appropriate vehicles for targeted drug delivery to infected MNPS cells, like the case of alveolar macrophages where *Mycobacterium tuberculosis* resides often. However, the uptake by the MNPS cells decreases liposomes half-life and exposes to high risk of therapeutic failure when the site of interest is beyond the MNPS [2].

Extensive studies conducted in liposome technology led to identification of some astute strategies for addressing the issue of MNPS' attack viz., liposome downsizing and surface modification. In this regard, it was observed that the physiological clearance of larger liposomes (MLV) was much quicker than that of smaller liposomes (SUL), which describe long-circulating profiles with increased half-lives [30]. The stealth strategy arising from surface modification involved grafting or coating hydrophilic polymers such as polyethylene glycol (PEG) and chitosan, which prevents detection of liposomes by the MNPS cells. While stealth behavior allows liposomes to achieve much longer circulation time (hence the name "long circulating liposomes"), this strategy comes along with poor targeting efficiency due to wider distribution of liposomes in the body. From this limitation, further developments have led to introduction of targeted liposomes. These liposomes are characterized by surface decoration with glycoproteins, polysaccharides or specific receptors ligands to achieve narrowed distribution and accumulation at the site of interest [9, 31]. The observation that ligand-decorated liposomes could provide selective drug accumulation inspired further design of antibody-functionalized liposomes (immunoliposomes) as well as stimuli-responsive liposomal systems for controlled drug delivery [32]. **Figure 4** shows the trend in the development of "intelligent" liposomes for site-specific delivery; from conventional liposomes, stealth liposome, targeted liposomes, immunoliposomes to stimuli-responsive liposomes.

Stimuli-responsive liposomes are smart liposomal systems that exhibit rapid release of the cargo upon changes in some physicochemical or biochemical stimuli, such as pH, temperature, redox potentials, enzymes concentrations, ultrasound, electric or magnetic fields [33, 34]. Among these stimuli, pH change is the most promising stimulus due to the existence of multiple pH gradients in the body [35]. In common practice, formulation of pH-sensitive liposomes involves incorporation of CHEMS and DOPE. Although CHEMS-DOPE-based liposomes have shown great promise for controlled delivery [24, 25, 36], the costly status of these lipids remains a deep concern for wider development and application of liposome products. To circumvent the use of such special lipids for pH-dependent delivery, our group has considered hydrazone derivatization of isoniazid (INH), as a small hydrophilic drug model, followed by encapsulation using crude soybean lecithin for cost-effective development [37]. Being poorly water soluble, the prepared conjugates were successfully embedded within the lipid membranes and INH release experiments were conducted in different pH media. The INH-conjugate loaded crude soybean lecithin



**Figure 4.** Schematic representation of the trend in liposome improvements for site specific delivery. (A) Conventional liposomes, (B) stealth liposome, (C) targeted liposomes, (D) immunoliposomes and (E) stimuli-responsive liposomes [32].

liposomes have demonstrated attractive nanoparticulate and stimuli-responsive characteristics for potential low-cost site-specific liposomal delivery [38]. However, the amounts of INH-derivatives loaded were found to be almost 10-folds lesser than the loading achieved when native INH was encapsulated in crude soybean lecithin liposomes, which was in turn associated with some burst release [29]. This is probably due to the fact that INH derivatives are encapsulated in the lipid bilayer, which offers limited space for loading [39], while the native INH was trapped within the aqueous core of liposomes. These limitations underline the need for further developments in liposome technology to achieve controlled release from cost-effective liposomes, combining both use of cheap lipids and high drug loadings.

## 4. Liposomes preparation and characterization

### 4.1 Preparation methods

Liposomes can be prepared using a wide range of methods that involve combination of lipids with aqueous media, and somehow affect liposomes characteristics, such as size, lamellarity and encapsulation efficiency (EE). The recently reported methods can be categorized as conventional, which mostly involve approaches that are easy to use at laboratory scale, and novel methods that appear to be more useful for up-scale production but require some special equipment [4].

#### 4.1.1 Conventional methods

The most commonly used methods for formulation of liposomes share the following fundamental stages: (i) lipids dissolution in organic solvents, (ii) drying of the resultant solution, (iii) hydration of dried lipid (using various aqueous media), (iv) isolation of the liposomal vesicles, and (v) quality control assays [6]. While sharing these basic stages, the conventional preparation methods gather different advantages and disadvantages that are comparatively presented in **Table 3**. The specific technological details of these methods are separately discussed in the following paragraphs.



Method designation	Advantages	Disadvantages
Film hydration (Bangham method)	Straightforward process	Use of organic solvent and mechanical agitation, production of large particles with no control on size, poor encapsulation efficiencies of hydrophilic materials, time consuming, sterilization issue
Reverse phase evaporation	Simple design, suitable encapsulation efficiency	Not applicable to fragile cargoes, use of large quantity of organic solvent, time consuming, sterilization issue
Solvent injection	Straightforward approach	Trace of organic solvent as residue, possible nozzle blockage in ether system, time consuming, sterilization issue
Detergent removal	Simple design, homogenous product, control of particle size	Presence of organic solvent, detergent residue, time consuming, low entrapment efficiency, poor yield, sterilization required
Heating method	Simple and fast process, organic solvent free, no need for sterilization, possible up-scale production	The need for high temperature

*Adapted from [40].*

**Table 3.**  
*Advantages and disadvantages of conventional methods.*

#### 4.1.1.1 Film hydration

Also known as the Bangham method, film hydration represents the simplest and oldest method used in liposome technology. In this method, lipids are firstly dissolved in a suitable organic solvent, and dried down to yield a thin film at the bottom of the flask. The obtained lipid film is hydrated using an appropriate aqueous medium to produce liposomal dispersion. The structural organization of the formed vesicles can be affected by the hydration conditions. A gentle hydration of the lipid film forms giant unilamellar vesicles (GULV), whereas a harsh hydration gives rise to multilamellar vesicles (MLV) with poor size homogeneity, which requires an additional downsizing step. The most commonly used sizing methods include probe and bath sonication that afford production of small unilamellar vesicles (SUV). Despite its higher effectiveness, probe sonication is often blamed for potential contamination (with titanium from the titanium-based nozzle used for mechanical agitation), and production of local heat that can affect lipids and drugs stability. Although the two sonication methods produce liposomes with identical characteristics, the use of bath sonication remains a better option due to easy control of operational parameters. Another technique used for liposome sizing includes consecutive extrusion of the liposomal formulation through polycarbonate filters of defined pore sizes. In this method, the number of extrusion cycles is the key parameter to control for effective homogenization [4, 6].

#### 4.1.1.2 Reverse phase evaporation

Reverse phase evaporation is an alternative method to the film hydration that involves formation of water-in-oil emulsion between the aqueous phase (containing hydrophilic materials) and the organic phase (containing lipids and any hydrophobic materials). A brief sonication of this mixture is required for system homogenization. The removal of the organic phase under reduced pressure yields a milky gel

that turns subsequently into liposomal suspension. The liposomes can be isolated from the dispersion using centrifugation, dialysis or sepharose 24 column [28].

#### *4.1.1.3 Solvent injection*

Solvent injection involves quick injection of the lipid solution (in ethanol or diethyl ether) into an aqueous medium. The experiment is performed either at room or at higher temperature (e.g., 60°C), depending on whether the organic solvent is water-miscible or not. The liposomes prepared by solvent injection process are mostly polydispersed and highly contaminated by organic solvents, especially ethanol due to formation of azeotrope mixture with water. As presented in **Table 3**, solvent injection suffers from several drawbacks including continuous exposure of the therapeutic agents to high temperature and organic solvent that might affect both the stability and safety of the liposomal products [28, 40].

#### *4.1.1.4 Detergent removal*

In the detergent removal method, phospholipids are dissolved in aqueous solution containing detergents at critical micelle concentrations (CMC). Upon detergent removal, the reaction medium frees individual phospholipid molecules that self-assemble into bilayered structures. Detergent removal is mostly achieved by means of a dialysis bag, polystyrene-based absorber beads or Sephadex columns (gel permeation chromatography). Dilution of the resultant mixture with some appropriate aqueous medium leads to restructuration of the formed micelles that evolve to liposomes [4, 6].

#### *4.1.1.5 Heating method*

Among all the conventional methods, the heating method is known to be the most attractive method for liposomes preparation due to its organic solvent free characteristics. In the heating method, lipids are hydrated for 1 hour, and heated for another hour above the transition temperature of the phospholipids in the presence of a hydrating agent (glycerin or propylene glycol 3%). When cholesterol is part of the formulation, the reaction medium is heated up to 100°C because of its high melting point. Being prepared under heating conditions, the resultant liposomes can be readily used without any further sterilization treatments, which minimizes both formulation complexity and timing. In addition, there is no need for further removal of the hydrating agents employed, since these represent physiologically acceptable ingredients that are well-established for pharmaceutical applications. Moreover, the observation that these hydrating agents can prevent particle coagulation and sedimentation makes them much more attractive as stabilizer and isotonic additives. The hydroxyl groups of these hydrating agents provide a cryoprotective effect that makes the heating method an efficient method for the formulation of inhalable liposomes [41, 42].

### *4.1.2 Novel preparation methods*

#### *4.1.2.1 Microfluidic channel method*

Microfluidic methods include all the novel techniques that make use of microscopic channels (in the size range of 5–500 µm). In this method, lipids are dissolved in an appropriate organic solvent (ethanol or isopropanol) and the resultant solution is propelled perpendicularly or in the opposite direction to the aqueous

medium within the micro-channels. The continuous axial mixing of the organic and aqueous solutions leads to liposomes formation because of local diffusion of phospholipids in aqueous phase, which encourages the self-assembly process. Among many others, the micro hydrodynamic focusing method represents the most commonly used microfluidic method for liposomes formulation. This method produces small and large unilamellar vesicles, 40–140 nm, with good size homogeneity (mono dispersed feature). The other microfluidic techniques include the microfluidic droplets and the pulsed jet flow microfluidic methods. The microfluidic droplets method involves dissolution of phospholipids in hexane for preparation of giant liposomes (4–20  $\mu\text{m}$ ). In the pulsed jet flow microfluidic method, the conventional film hydration method has been modified by drying the lipid solution in microtubes. The resultant lipid film is hydrated within the microtubes through a perfusion process that produces much larger vesicles, 200–534  $\mu\text{m}$ , with remarkable encapsulation efficiency [4, 43]. As common advantages, the microfluidic methods offer the possibility for production of vesicles with desired size, due to the versatility and flexibility of the methods. The disadvantages of these methods include the imperative use of organic solvent and drastic agitation, as well as difficulty for large scale production [40].

#### *4.1.2.2 Supercritical fluidic method*

While being considered as equivalent to the conventional reverse phase evaporation method, supercritical fluidic technique represents the most important novel liposome preparation methods that makes use of a supercritical fluid, such as carbon dioxide ( $\text{CO}_2$ ) maintained under supercritical conditions (supercritical temperature and pressure). In this state,  $\text{CO}_2$  is an excellent solvent for the lipids. The high-performance liquid chromatography (HPLC) pump provides a continuous flow of the aqueous phase into a view cell that contains the supercritical lipid solution, allowing phase transition of the dissolved phospholipids. Upon sudden decrease in pressure,  $\text{CO}_2$  gets completely removed and phospholipids self-assemble into a bilayered vesicular system. The supercritical fluidic method affords large unilamellar vesicles (100–1200 nm) with 5-fold higher encapsulation efficiency than the equivalent conventional method. Apart from being organic solvent-free methods, the supercritical fluidic method offers many other advantages such as the use of  $\text{CO}_2$ , as a cheap and environmentally harmless solvent, possibility for controlling particle size, in situ sterilization and large-scale production in industrial settings. However, the disadvantages of the supercritical fluidic technique, including particularly its high cost, low yield and use of high pressures (200–350 bar) which require special infrastructures, restrict their universal applications for wider developments of liposomal technology [4, 40, 43].

#### *4.1.3 Post-preparation treatments*

##### *4.1.3.1 Freeze-thawing*

The freeze-thawing treatment involves freezing the liposomes dispersion in liquid nitrogen, and subsequently thawing it at the temperature above the phase transition temperature of the lipids used for formulation. Upon freeze-thawing, the liposomal vesicles are subjected to fusion since the lipid bilayers become fluid and highly permeable, allowing extensive diffusion of hydrophilic molecules, which leads to important cryoconcentration. These structural modifications encourage encapsulation of hydrophilic materials that are poorly loaded in liposomes when conventional methods are used. This underlines the reason why freeze-thawing

represents an important treatment in liposome technology. Amongst the key parameters to be considered for freeze-thawing optimization are the number and duration of freeze-thawing cycles. These can impact significantly not only the encapsulation efficiency but also structural characteristics, i.e., liposomes lamellarity and polydispersity [44, 45].

#### 4.1.3.2 Freeze-drying

Commonly known as lyophilization, freeze-drying is a post-preparation treatment for liposomes that is applied in both laboratory and industrial settings to preserve the characteristics of liposomal products. Freeze-drying involves freezing of the aqueous samples and subsequent removal of ice by sublimation. Freeze-drying represents a very useful treatment for shelf stability of liposomal suspensions, since water molecules can trigger some chemical reactions and lead to modification of the cargo or excipients in the formulation. Freeze-drying appears to be of great interest when the prepared formulation contains thermos-sensitive materials such as proteins, nucleic acids, etc., which might undergo fast degradation when subjected to heat-drying. The use of freeze-drying has gained considerable attention in liposome technology due to improved storage stability of liposomal products. Because of potential leakage of liposomes during freeze-drying, addition of hydrophilic compounds, commonly called cryoprotective agents (such as carbohydrates), has been established to ensure good stability and quality of the final product. The cryoprotectants commonly used include mannitol, lactose, sucrose and trehalose. Among these, trehalose is the most reputed cryoprotecting agent since it preserves liposomes stability during and after freeze-drying treatment [6].

## 4.2 Characterization techniques

After production, liposome formulations are subjected to extensive characterization, evaluating the physicochemical properties of liposomes that affect their shelf stability and biological performance. The most routinely investigated parameters in liposome characterization include vesicle size and size distribution (or polydispersity), surface charge (or Zeta potential), shape and morphology, lamellarity, encapsulation efficiency, phase behavior (or polymorphism) and in vitro release profile [4, 46]. **Table 4** indicates the techniques used for evaluation of liposome characteristics. The most frequently used methods are briefly discussed in the following paragraphs.

### 4.2.1 Dynamic light scattering (DLS)

Also known as photon correlation spectroscopy or quasi-elastic light scattering, DLS represents the most commonly used method for determination of liposome size, size distribution (polydispersity) and Zeta potential (surface charge). DLS is done by an instrumental setting called Zetasizer Nano. The standard operational principle of DLS is based on continuous motion of dispersed particles due to their bombardment by solvent molecules (Brownian motion). This phenomenon causes remarkable scattering of the applied light. Since the extent of fluctuation in light intensity is associated with the diffusion rate of the suspended particles, which is related to particle diameter (smaller particles diffusing faster than the larger ones), the particle size is automatically deducted from the estimated amount of the scattered light. When addressing Zeta potential measurements, DLS allows surface charge determination by accessing changes in the scattered light intensity caused by particle motion due to the electric field applied. In other words, for surface



Parameters	Analytical techniques
Particle size	Dynamic light scattering (DLS), size exclusion chromatography (SEC), field-flow fractionation (FFF) and microscope technology: transmission electron microscopy (TEM), cryogenic-TEM (Cryo-TEM), and atomic force microscopy (AFM)
Zeta potential /Surface charge	Electrophoretic mobility, DLS
Particle shape / morphology	Microscopic techniques such as TEM, Cryo-TEM and AFM
Lamellarity	Cryo-TEM and <sup>31</sup> P-NMR
Phase behavior	X-ray diffraction (XRD), differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)
Encapsulation efficiency	Centrifugation, dialysis or column separation for liposomes isolation, followed by drug content determination
Drug release	Dialysis or centrifugation, followed by drug quantification using analytical method, such as UV-Vis spectrophotometry, fluorescence spectrometry, enzyme- or protein-based assays, gel electrophoresis, HPLC, UPLC, LC-MS

**Table 4.**  
*Analytical methods commonly used for liposomes characterization.*

charge (Zeta potential) evaluation, changes in the intensity of the scattered light are governed by the applied electric field (which causes extensive motion of charged particles), in contrast to size measurements where Brownian motion is the key factor [4, 47]. Apart from being a simple, fast and reliable method for routine analyses, DLS offers many other advantages including the fact that the measurement is taken from a native environment, and a wide size range can be evaluated (from a few nanometers to several micrometers). However, DLS shows some limitations such as the difficulty of differentiating individual particles from aggregates and high sensitivity to contaminants [48]. In addition, DLS is technically unable to provide true particle size, but rather hydrodynamic diameter due to particle solvation. Water layers on particle surface lead to false readings of particle diameters in aqueous media [49].

#### 4.2.2 Transmission electron microscopy (TEM)

The microscopic observation provides direct visualization of the liposomal vesicles as individual particles, which allows effective analysis of shape and morphology as well as a precise and reliable size reading. In this context, TEM techniques are commonly used in liposome technology for structural characterizations. In TEM experiments, the liquid sample is spotted onto a copper grid, and the solvent dried prior to the microscopic analysis. Under TEM instrument, liposomal vesicles mostly appear as black spherical particles on a white background. For a variant TEM technique like negative staining TEM, liposomes appear as bright spherical spots on a black background since the spotted sample is treated with uranyl acetate or phosphotungstic acid (as negative staining agent). Due to its effectiveness, TEM appears to be a powerful complementary technique to DLS for confirmation of the liposomal structure. Unlike DLS, TEM offers the advantage of differentiating individual vesicles from aggregates, allowing critical assessment of the liposome population. Nevertheless, TEM presents several limitations due to sample preparation. Apart from being time-consuming, sample pretreatments in TEM analyses may cause remarkable changes in liposomal shape or morphology: potential vesicle shrinkage, swelling or artifact formation [4, 50].

To overcome these limitations, Cryo-TEM was developed as a strategy to minimize liposome disruption by making use of a flash freezing treatment for direct particle visualization in solid-state (without solvent removal). Nowadays, Cryo-TEM is the most reliable technique for visual determination of liposome structure including lamellarity. However, Cryo-TEM appears to be sometimes limited since it works perfectly only with very small particles. This has led to the development of atomic force microscopy (AFM) for direct particle analysis in native environments. Although AFM offers the advantage of higher particle resolution at three-dimensional level, the use of this technique is mostly limited by the high cost of the instrument, which compromises its universal availability and accessibility [3, 4].

#### *4.2.3 Differential scanning calorimetry (DSC) and X-ray diffraction (XRD)*

DSC and XRD are complementary techniques that evaluate the thermal behavior and crystallinity, respectively, and provide valuable information for characterization of loaded liposomes [46, 51, 52]. DSC evaluates the differences in heat flow (electric power) between a sample and a reference. In DSC experiments, the sample and the reference are subjected to a programmed heating, cooling or isothermal treatment in a controlled atmosphere (mostly saturated with nitrogen gas). The heating treatment is achieved either by the same heater (heat flux DSC) or by separate heaters (power compensated DSC). The experiment is conducted in specialized metal pans made of aluminum, tin, zinc or indium. Throughout the experiment, frequent electric power adjustments occur upon material phase transition (melting or crystallization), ensuring thermal equilibrium between the sample and the reference. This phenomenon is described and expressed by the plot of heat capacity against temperature or time (heat flow curve). The heat flow curve provides the respective transition temperature and enthalpy, which allows to identify the nature of thermal events: endo- or exothermic [53–55]. DSC represents the most useful thermal analysis technique in the study of lipid-based materials [46, 52, 54–56].

Unlike DSC where sample recovery is not possible, XRD is a non-destructive analytical tool that allows structural investigations of crystalline materials. XRD makes use of X-rays that deeply penetrate solid materials and provide useful information at atomic structure level. Though relatively expensive, an XRD instrument is an environmentally and user-friendly device that is easy to use. A wide range of materials such as powders, crystals and liquids can be quickly assessed by XRD. Its other advantages include high resolution, reliability, relatively cheap maintenance, and easy data collection, processing and interpretation. The phase transitions and polymorphism determined by XRD represent valuable information in pharmaceutical development and production of both excipients and biologically active materials [57–59].

#### *4.2.4 Lamellarity assays*

The lamellarity of liposomes is part of their structural characteristics that can have an impact on the intended applications. The number of lipid bilayers can be evaluated using chemically labeled or radiolabelled agents that can be distributed in the bilayer membranes. However, this technique is limited since these reagents might be distributed only on the outer lipid membrane and lead to false readings. To date, Cryo-TEM is the most commonly used technique for the determination of lamellarity by visualization [4]. The nuclear magnetic resonance spectroscopy of the 31-phosphorus ( $^{31}\text{P}$ -NMR) is also being currently used to estimate the lamellarity of liposomes. This technique deals with the estimation of the ratio of phospholipid amount in the outer layers to that of the inner layers [60]. The  $^{31}\text{P}$ -NMR spectrum with a broad peak indicates the presence of MLV while a narrow peak

corresponds to SUV. The addition of paramagnetic ions such as  $Mn^{2+}$ ,  $Co^{2+}$ , and  $Pr^{3+}$  shifts the respective peaks to either downfield or upfield because of ionic interactions with the phosphate backbone. By comparing the spectroscopic profile with and without the paramagnetic ion, the lamellarity of liposomes can be estimated. Some other techniques such as small-angle X-ray scattering (SAXS) and trapped volume determination can be used to estimate liposome lamellarity [4, 61].

#### *4.2.5 In vitro release assays*

The profile of release for the liposomal cargoes is commonly estimated in vitro using dialysis. This method implies trapping the liposomal dispersion into a dialysis bag of specific molecular weight cut off. The resultant tubing membrane is placed in a simulated physiological fluid (release medium) that is often a buffer maintained under well-defined conditions: specific temperature and speed of stirring/shaking. At predetermined time intervals, an aliquot is withdrawn from the release medium and an equal volume of the fresh buffer is replaced to maintain sink conditions. In the withdrawn sample aliquots, the released cargo is quantified using some routine analytical techniques such as UV-Vis spectrophotometry, HPLC, UPLC, etc., adapted to the molecular species under evaluation. The release profile is obtained by plotting the cumulative release percentage against the chosen time intervals [4]. Data from the in vitro release study are valuably considered as part of the rational development of formulations for controlled release, since they allow effective prediction of in vivo performance of the delivery systems [62].

## **5. Applications**

Liposomes have evolved so far from mere experimental tools of research to industrially established products for clinical and veterinary use. They have shown the ability to improve the physicochemical features of the cargoes and ferry them to the sites of interest. The concepts of liposomal encapsulation have been applied in several fields of life science. Liposomes are frequently used for the delivery of drug, gene, vaccine and diagnostic products; but other applications encompass encapsulation of food and cosmetic ingredients as well as routine analysis of chemical substances [2, 3]. The following paragraphs briefly present the current applications of liposomes.

### **5.1 Application in drug delivery**

The use of liposomes in drug delivery aims at modifying the pharmacokinetics of drugs to improve the therapeutic efficacy while minimizing potential toxicity [6]. Liposomes can alter the spatial and temporal distribution of the entrapped drug molecules in vivo, leading to controlled delivery at the site of interest and reduced off-target adverse effects [63]. The liposomal systems have been extensively investigated for the delivery of existing and emerging drugs at various research levels, from basic stages related to research and development to preclinical and clinical applications. Nowadays, liposomes represent the most clinically established drug vehicles for human diseases [3, 5]. The efforts invested in liposomal technology have so far led to the development of several effective liposomal formulations that are currently used in clinics (**Table 5**).

Liposomal formulations have been used to address a wide range of pathological conditions through different administration routes, including dermal, transdermal, oral, pulmonary and parenteral routes. The clinical areas commonly explored in liposome research encompass skin disorders, cancers and infectious diseases [4].

Branded product	Drug name	Therapeutic indications
Abelcet	Amphotericin B	Fungal infections
AmBisome	Amphotericin B	Fungal infections
Amphocil	Amphotericin B	Fungal infections
DaunoXome	Daunorubicin	Hematological malignancy
DepoCyt	Cytarabine	Lymphomatous meningitis
DepoDur	Morphine sulfate	Pain relief
Doxil	Doxorubicin	Kaposi's sarcoma and solid tumors
Epaxal	Inactivated hepatitis A virus	Hepatitis A
Evacet	Doxorubicin	Ovarian cancer
Inflexal V	Inactivated hemagglutinin of influenza virus strains A and B	Influenza
LipoDox	Doxorubicin	Kaposi's sarcoma and solid tumors
Marqibo	Vincristine sulfate	Acute lymphoblastic leukemia
Visudyne	Verteporfin	Photodynamic therapy

**Table 5.**  
*Clinically approved liposomal products [3, 4].*

Amongst, cancer therapy appears to be in the forefront of liposome delivery, due to poor bioavailability and side effects of most of the anti-cancer drugs. However, several infectious diseases, most specially where the pathogen is hosted by the MNPS (i.e., tuberculosis, leishmaniasis, fungal infections), have been reported to be good candidates for liposome application, taking advantage of the spontaneous liposomes uptake by the cells of MNPS [2]. Apart from the nature and localization of the disease, the design and development of liposomes depend also on the intended administration route, since different anatomical and physiological characteristics can be encountered from one route to another [46].

Furthermore, the application of liposomes in drug delivery is highly dependent on their colloidal and physiochemical features, i.e., vesicle size, surface charge and system stability [64]. For instance, small liposomes (SUV) are good candidates for Parkinson's and Alzheimer's diseases, due to the need for crossing the brain blood barrier to achieve brain targeted delivery. Meanwhile, large liposomes are preferred for macrophage targeted delivery of antimicrobials, when pathogens are located inside the MNPS cells (e.g., tuberculosis, leishmaniasis), taking advantage of the passive liposome cell uptake [2, 4, 46]. This underlines the need for thorough exploration of process and formulation parameters at early stages of products development to produce liposomes with desired characteristics, making the technology for liposomes manufacturing key to future therapeutic research and development.

## 5.2 Application in gene delivery

Liposomes have been reported to achieve effective intracellular delivery of genes. These liposomes, also called lipoplexes, are generally made of cationic lipids, which allow for encapsulation of genetic materials via electrostatic interactions with the negatively charged phosphate backbones of nucleic acids. The positive charge on the surface of liposomes also influences their interactions with negatively charged cell membranes and promotes cell internalization [23, 65]. Cationic liposomes offer several advantages over viral gene vectors, including easy and safe production,



cost effectiveness, possibility of monitoring toxicity, biodegradability, biocompatibility and lack of dangerous immunogenicity. However, they are suffering from poor transfection efficiency due to their limited endosomal escaping ability, which exposes the genetic materials to enzymatic and acid degradation in lysosomes. This has been improved by incorporating fusogenic lipids, such as DOPE, in the liposomal formulation. These helper lipids facilitate endosomal escaping by membrane fusion with endosomes, leading to early cytoplasmic release of the gene. Though cationic liposomes have shown some dose-dependent toxicity, successful results have been obtained in cancer therapy when delivering genes encoding for tumor suppression proteins [66, 67]. He et al. have recently developed folate receptor alpha-targeted lipoplexes with therapeutic gene expression regulated by an hTERT promoter. These liposomes have shown some promise for the treatment of ovarian cancer [68].

### **5.3 Application in vaccine delivery**

The use of specific lipids or molecules such as phosphatidylserine, DOTAP, fatty acids and monophosphoryl lipids can produce liposomes with attractive immune-stimulating activities. Liposomes carrying antigenic materials, either encapsulated in the aqueous core, grafted or coated on the surface; can stimulate immune responses on macrophage uptake. Following endolysosomal degradation, macrophages present the antigen to T-lymphocytes that initiate the production of cytokines and specific antibodies via activation of B-lymphocytes [4]. The immune response produced by liposomes can be influenced by their composition, lamellarity, size and surface charge [69]. Liposomes containing a glycolipid, trehalose 6,6'-dibehenate, and a cationic lipid, dimethyldioctadecylammonium, in a 1:5 mass ratio have demonstrated efficient delivery of the TB vaccine Ag85B-ESAT-6. This vaccine has shown prolonged immune response without any toxic effects [70]. Although a liposomal vaccine (namely Stimuvax<sup>®</sup>) targeting the major histocompatibility class I complex for lung carcinoma was not successfully implemented, some other vaccines such as Epaxal<sup>®</sup>, Inflexal<sup>®</sup> V and Mosquirix<sup>®</sup> have been clinically established for the treatment of hepatitis A, influenza virus infections and malaria, respectively. These vaccines, classified as virosomes, are liposomes generally made of reconstituted viral envelop supplemented with phosphatidylcholine. They offer the advantage of undergoing membrane fusion either with the cells or the endosomes, and thus leading to efficient cytosolic delivery [2, 9].

### **5.4 Application in diagnosis**

The use of liposomes for diagnostic purposes is one of current topics of great interest in biomedical applications. Liposomes with magnetic properties, also called magnetoliposomes, are made by entrapping superparamagnetic iron-based nanoparticles or iron oxides or gadolinium (III) chelates for magnetic resonance imaging. Functionalized liposomes labeled with radioisotopes can also be used as molecular probes in nuclear imaging. For instance, liposomes labeled with <sup>64</sup>Cu, <sup>18</sup>F, <sup>89</sup>Zr or <sup>52</sup>Mn have been reported in positron emission tomography while <sup>99m</sup>Tc, <sup>111</sup>In or <sup>67</sup>Ga labeled liposomes were applied in single photon emission computed tomography. Acoustic liposomes which are liposomes made of perfluoropropane gas can be used as contrast agents in ultrasound imaging technique. The encapsulation of quantum dots and fluorescent dyes into liposomes has also led to the development of attractive liposomal platforms for diagnosis. Additionally, liposomes have shown great potential for simultaneous accommodation of drugs and diagnostic agents such as radionuclides, magnetic or contrast substances. This can be achieved by encapsulation in the inner core, embedding in the lipid bilayer, chemically grafting

or coating onto the surface of liposomes. A successful targeted co-delivery of these materials has given to liposomes the status of theranostic systems, as they provide both the therapeutic effect and the diagnostic control [2, 71, 72].

### **5.5 Application in analytical fields**

In analytical domains, liposome-based formulations can be usefully involved in immunoassays, biosensors analysis and liquid chromatography. Liposomes can be used to encapsulate, embed or conjugate the analytical entities with high loading capacity and huge surface area that can enhance the intensity of analytical signal. In a direct enzyme-linked immunosorbent assay (ELISA) like method, fluorophore-conjugated liposomes carrying a substantial amount of secondary antibody quickly bind to the antigen that has been fixed on the primary antibody. The addition of a colorimetric substrate leads to color development and allows for analytical estimation of the antigenic analyte. The use of liposomes has been deemed to lower the limits of detection of analytes and increase the sensitivity of immunological analysis technique [73]. In chromatography, the conjugation of liposomes to the stationary phase for gel permeation chromatography is useful for separation of drugs and proteins and for exploring possible molecular interactions on phospholipid membranes. Additionally, the cell-like appearance of liposomes makes them appropriate simulated cell models for studying and predicting the interactions between biologically active compounds and cell membranes [4].

### **5.6 Application in cosmetics**

The use of liposomes in the field of cosmetics is based on the similarity between the lipid composition of the liposomal vesicle and that of the biological layers composing the skin. Because phospholipids can be subject to hydration, topical liposomes happen to contribute to the reduction of skin dryness. While playing the role of attractive vehicles for relevant cosmetic agents, liposomes provide a great source of skin ingredients, such as essential unsaturated fatty acids like linolenic acid. This has shown great potential for maintaining the skin and hair in good physiological standing, preventing the rise of some common topical diseases. Furthermore, the use of skin care formulations made of empty or hydrating agents loaded liposomes helps to reduce the transdermal water loss, which is a major cause of skin dryness [74, 75].

### **5.7 Application in food industry**

As versatile lipid-based systems, liposomes have shown some potential in the encapsulation of food ingredients. In this field, the use of liposomes aims to stabilize some nutraceutical or dietetic ingredients during the storage, to improve their organoleptic characters or to provide a controlled and targeted delivery of these substances in a specific tissue. The shelf life and efficacy of instable bioactive products such as vitamins, enzymes and anti-oxidative agents have been prolonged by their liposomal encapsulation. For example, proteinase and lipase loaded liposomes have been reported to improve the ripening of cheese notably in preventing proteolysis of casein [28, 76]. Yokota et al. [27] have successfully improved the taste and odor of casein hydrolysate in dietetic preparations by liposomal encapsulation. In addition to the encapsulation of dietetic compounds, liposomes have been recently used to encapsulate a cyanobacterium, namely *Spirulina platensis*, as a source of proteins [77]. Although the use of liposomes in food industry is still at the infant stages of development, advanced studies have been launched to investigate much more attractive applications, such as detection of food contaminants by means of stimuli-responsive liposomes for food safety [76].

## 6. Conclusion

Liposomes appear to be reputed carriers for various chemical and macromolecular species. From way back their discovery, liposomes have been subject to extensive evolution, in terms of composition, manufacturing and usages, which led to several openings in both basic and applied life sciences. The general details presented herein attempted to reveal some of the existing gaps in liposomes technology and open new windows for further research. An important future breakthrough could be the discovery of cost-effective materials to formulate liposome vesicles with remarkable versatility viz., being suitable as vehicles for various molecules while holding a clear molecular architecture to act as cell models for in vitro bioassays. In addition, novel manufacturing methods for facile encapsulation of both hydrophobic and hydrophilic molecules with no need for organic solvents and special equipment or sophisticated infrastructures are highly desired. Furthermore, the critical issue of system instability for liposomes loaded with hydrophilic materials requires new strategies that will achieve acceptable loading while aiming at targeting cargo delivery at the site of interest. Finally, since liposomes have demonstrated some clinical success as drug vehicles, future efforts should be dedicated to ensuring wider developments and implementation of therapeutic liposomes. This would enhance commercial availability and accessibility of liposome products across the globe, particularly in low- and middle-income countries.

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## Conflict of interest

There is no conflict of interest to declare.

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