Liposomes, spherical-shaped nanovesicles, were discovered in the 60ies by Bangham. Since that, they were extensively studied as potential drug carrier. Due to their composition variability and structural properties, liposomes are extremely versatile leading to a large number of applications including pharmaceutical, cosmetics and food industrial fields. This bibliographic paper offers a general review on the background and development of liposomes with a focus on preparation methods including classic (thin film hydration, reverse-phase evaporation, ethanol injection…) and novel scalable techniques. Furthermore, liposome characterization techniques including mean size, zeta-potential, lamellarity, encapsulation efficiency, \textit{in vitro} drug release, vesicles stability and lipid analysis synthesized from different published works are reported. The current deepening and widening of liposome interest in many scientific disciplines and their application in pharmaceutics, cosmetics and food industries as promising novel breakthroughs and products were also handled. Finally, an opinion on the usefulness of liposomes in various applications ranging from unsubstantiated optimism to undeserved pessimism is given. The obtained information allows establishing criteria for selecting liposomes as a drug carrier according to its advantages and limitations.

\textbf{Keywords:} Liposomes, Preparation Methods, Characterization, Phospholipids, Therapeutic Application, Cosmetic, Food.

\section*{1. INTRODUCTION}
Liposomes, defined as microscopic spherical-shaped vesicles, consist of an internal aqueous compartment entrapped by one or multiple concentric lipidic bilayers. Liposomes membrane is composed of natural and/or synthetic lipids which are relatively biocompatible, biodegradable and non-immunogenic material. Because of their unique bilayer-structure properties, liposomes are used as carriers for both lipophilic and water-soluble molecules. Hydrophilic substances are encapsulated in the interior aqueous compartments. Lipophilic drugs are mainly entrapped within lipid bilayers.

As asserted by different authors, liposomes have attractive biological properties, including the biocompatibility and biodegradability. They show promise as active vectors due to their capacity to enhance the encapsulant performance by increasing drug solubility, and stability; delivering encapsulated drugs to specific target sites, and providing sustained drug release.\textsuperscript{1} Their sub-cellular size allows relatively higher intracellular uptake than other particular systems; improving \textit{in vivo} drug bioavailability.
A. Laouini is a Ph.D. student at the University of Lyon (France). His thesis is dealing with the application of membrane contactors for the preparation of colloidal systems. Prior to joining the University of Lyon, he received an M.Sc. in pharmacoctehnie in 2010 and a degree of pharmacist in 2007 from the University of Monastir (Tunisia). His Current research interests focus on developing advanced drug carriers (liposomes, micelles, nanoemulsions...) by the use of membranes which allows reproducible production process with less energy consumption.

C. Jaafar-Maalej after obtaining a Master degree in Biomedical engineering at the University of Lyon 1, France, Chiraz Jaafar Maalej earned his Ph.D. degree in pharmacoctehnie, with his thesis addressing the development of beclomethasone-liposomes suitable for pulmonary route. She is currently a postdoc fellow at the “Laboratoire d’Automatique et de Génie des Procédés” (LAGEP). Current research is directed toward the development of novel drug delivery systems for pediatric application from preformulation to in vivo pharmacokinetic studies in rats.

I. Limayem-Blouza after obtaining a Master degree in Biomedical engineering at the University of Lyon 1, France, Iméne Limayem Blouza obtained her Ph.D. degree (2006 at the University of Lyon 1) in pharmacoctehnie, with her thesis addressing the development of drug carriers (nano-particles) by the use of membrane contactor. She is actually aggregate professor in galenic pharmacy at the Faculty of Pharmacy of Monastir (Tunisia).

S. Sfar is Pharm.D., Ph.D. in pharmaceutical sciences. Currently she is a research professor of technological pharmacy and she is responsible of a research unit for medicines development. Professor Souad Sfar has published on various aspects of pharmaceutical technology such as nanotechnology, bioavailability, pharmaceutical dosage forms, etc.

C. Charcosset graduated from Ecole Centrale de Lyon (France) in 1987. She obtained a Ph.D. in Compiègne (France) in 1990; she was then appointed as a postdoctoral fellow at MIT (Boston, USA) from 1990 to 1992. She obtained a Research Scientist position at CNRS, first in the “Laboratoire des Sciences du Génie Chimique” (LSGC, Nancy, France), from 1992 to 1994; then in “Laboratoire d’Automatique et de Génie des Procédés” (LAGEP, Villeurbanne, France). She has actually a Research Director position. Her past and current research deals with membrane and membrane processes, such as characterization of membranes by confocal microscopy, membrane chromatography, ultrafiltration and microfiltration, preparation of emulsions and particles using membranes, and membrane crystallization for biotechnological, pharmaceutical and environmental applications.
Other advantages of liposomes include high encapsulation efficiency in spite of drug solubility, low toxicity due to phospholipid content, drug protection against degradation factors like pH and light and the reduction of tissue irritation.

Liposomes have been extensively studied as drug carriers in the pharmaceutical and medical fields. Research has expanded considerably over the last 30 years, increasing applications area from drug and gene delivery to diagnostics, cosmetics, long-lasting immune-contraception to food and chemical industry. The superiority of liposomes as drug carriers has been widely recognized. Ten liposomal and lipid-based formulations have been approved by regulatory authorities and many liposomal drugs are in preclinical development or in clinical trials.

Several reviews about liposomes as drug delivery systems and specific application via oral, topical, pulmonary and ophthalmic route have been published. Clearly, within the frame of a single review paper it is impossible to address all the pertinent issues, this bibliographic paper attempt to review liposomes current technology with respect to numerous multidisciplinary applications. As a contribution for updating the state of knowledge, a focuses on liposomes preparation method and recent characterization techniques including mean size, zeta-potential, lamellarity, encapsulating efficiency, in vitro active release, stability and lipid analysis have been described as well as the most significant achievements and applications.

2. LIPOSOMES

2.1. Definition

Liposomes were first produced in England in the 60’s, by Bangham who was studying phospholipids and blood clotting. According to legend, he was experimenting with new laboratory equipment, and he made a noted observation about phospholipids forming closed multilamellar vesicle spontaneously in aqueous solution which took two years to be proved. The phospholipid reorganisation in aqueous solution is mainly driven by the hydrophobic effect which organizes amphiphilic molecules (phospholipids) so as to minimize entropically unfavorable interactions between hydrophobic acyl-chains and surrounding aqueous medium. This effect is further settled by various intermolecular forces such as electrostatic interactions, hydrogen bonding, as well as Vanderwaals and dispersion forces.

Liposomes were defined as an artificial microscopic vesicle consisting of a central aqueous compartment surrounded by one or more concentric phospholipid layers (lamellas) (Fig. 1). Furthermore, hydrophilic (in the aqueous cavity), hydrophobic (within lipidic membrane) and amphiphilic substances are able to be incorporated within these vesicles developing large potential applications. Numerous researchers have worked with these structures since Bangham’s discovery, making of liposomes the most popular nanocarrier systems.

2.2. Classification

Liposomes can be classified in terms of composition and mechanism of intracellular delivery into five types:

(i) conventional liposomes;
(ii) pH-sensitive liposomes;
(iii) cationic liposomes;
(iv) immunoliposomes and
(v) long-circulating liposomes.

Otherwise, vesicle size is a critical parameter in determining circulation half-life of liposomes, and both size and number of bilayers influence the extent of drug encapsulation within liposomes. Thus, liposomes were typically classified on the basis of their size and number of bilayers into (Fig. 2):

(i) Small unilamellar vesicles (SUV): 20–100 nm;
(ii) Large unilamellar vesicles (LUV): > 100 nm;
(iii) Giant unilamellar vesicles (GUV): > 1000 nm;
(iv) Oligolamellar vesicle (OLV): 100–500 nm and
(v) Multilamellar vesicles (MLV): > 500 nm.

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Fig. 1. Schematic drawing of liposomes structure and lipophilic or hydrophilic drug entrapment models.

Fig. 2. Liposomes classification based on size and lamellarity.
New developed types of liposome, designated as double liposome (DL)\textsuperscript{19} and multivesicular vesicles (MVV),\textsuperscript{20} were recently reported. These liposomes, which could be prepared by novel preparative technique, are thought to improve drug protection against several enzymes.\textsuperscript{21}

3. LIPOSOMES PREPARATION PROCEDURES

3.1. General Ingredients

Generally, liposome composition includes natural and/or synthetic phospholipids (Phosphatidylethanolamine, Phosphatidylglycerol, Phosphatidylcholine, Phosphatidylinositol) Phosphatidylcholine (also known as lecithin) and phosphatidylethanolamine constitute the two major structural components of most biological membranes. Liposome bilayers may also contain other constituents such as cholesterol,\textsuperscript{1} hydrophilic polymer conjugated lipids and water.

Cholesterol has been largely used to improve the bilayer characteristics of the liposomes. It improves the membrane fluidity, bilayer stability and reduces the permeability of water soluble molecules through the membrane.\textsuperscript{22}

A clear advantage of liposomes is the fact that the lipid membrane is made from physiological lipids which decreases the danger of acute and chronic toxicity.

3.2. Preparation Method

3.2.1. Classical Technique

There are four classical methods of liposome manufacture. The difference between the various methods is the way in which lipids are drying down from organic solvents and then redispersed in aqueous media.\textsuperscript{23} These steps are performed individually or are mostly combined.

3.2.1.1. Hydration of a Thin Lipid Film: Bangham Method. This is the original method which was initially used for liposome production.\textsuperscript{24} A mixture of phospholipid and cholesterol were dispersed in organic solvent. Then, the organic solvent was removed by means of evaporation (using a Rotary Evaporator at reduced pressure). Finally, the dry lipidic film deposited on the flask wall was hydrated by adding an aqueous buffer solution under agitation at temperature above the lipid transition temperature.

This method is widespread and easy to handle, however, dispersed-phospholipids in aqueous buffer yields a population of multilamellar liposomes (MLVs) heterogeneous both in size and shape (1–5 μm diameter). Thus, liposome size reduction techniques, such as sonication for SUVs formation or extrusion through polycarbonate filters forming LUVs\textsuperscript{25, 26} were useful to produce smaller and more uniformly sized population of vesicles.

3.2.1.2. Reverse-Phase Evaporation (REV) Technique. A lipodic film is prepared by evaporating organic solvent under reduced pressure. The system is purged with nitrogen and the lipids are re-dissolved in a second organic phase which is usually constituted by diethyl ether and/or isopropyl ether. Large unilamellar and oligolamellar vesicles are formed when an aqueous buffer is introduced into this mixture. The organic solvent is subsequently removed and the system is maintained under continuous nitrogen. These vesicles have aqueous volume to lipid ratios that are 30 times higher than sonicated preparations and 4 times higher than multilamellar vesicles. Most importantly, a substantial fraction of the aqueous phase (up to 62% at low salt concentrations) is entrapped within the vesicles, encapsulating even large macromolecular assemblies with high efficiency.\textsuperscript{27}

3.2.1.3. Solvent (Ether or Ethanol) Injection Technique. The solvent injection methods involve the dissolution of the lipid into an organic phase (etherol or ethanol), followed by the injection of the lipid solution into aqueous media, forming liposomes.\textsuperscript{28}

The ethanol injection method was first described in 1973.\textsuperscript{29} The main relevance of the ethanol injection method resides in the observation that a narrow distribution of small liposomes (under 100 nm) can be obtained by simply injecting an ethanolic lipid solution in water, in one step, without extrusion or sonication.\textsuperscript{30}

The ether injection method differs from the ethanol injection method since the ether is immiscible with the aqueous phase, which is also heated so that the solvent is removed from the liposomal product. The method involves injection of ether-lipid solutions into warmed aqueous phases above the boiling point of the ether. The ether vaporizes upon contacting the aqueous phase, and the dispersed lipid forms primarily unilamellar liposomes.\textsuperscript{31} An advantage of the ether injection method compared to the ethanol injection method is the removal of the solvent from the product, enabling the process to be run for extended periods forming a concentrated liposomal product with high entrapment efficiencies.

3.2.1.4. Detergent Dialysis. Liposomes, in the size range of 40–180 nm, are formed when lipids are solubilized with detergent, yielding defined mixed micelles.\textsuperscript{32} As the detergent is subsequently removed by controlled dialysis, phospholipids form homogeneous unilamellar vesicles with usefully large encapsulated volume.

Other methods have been already used for liposomes preparation such as: calcium induced fusion,\textsuperscript{33} nanoprecipitation,\textsuperscript{34} and emulsion techniques.\textsuperscript{35, 36}

However, these classical techniques require large amounts of organic solvent, which are harmful both to the environment and to human health, requiring complete removal of residual organic solvent. Furthermore, conventional methods consist of many steps for size homogenization and consume a large amount of energy which is unsuitable for the mass production of liposomes.
3.2.2. New Large-Scale Liposome Technique

Since industrial scale production of liposomes has become reality, the range of liposome preparation methods has been extended by a number of techniques such as Heating Method, Spray drying, Freeze drying, Super Critical Reverse Phase Evaporation (SCRPE), and several modified ethanol injection techniques which are increasingly attractive.

3.2.2.1. Heating Method. A new method for fast production of liposomes without the use of any hazardous chemical or process has been described. This method involves the hydration of liposome components in an aqueous medium followed by the heating of these components, in the presence of glycerol (3% v/v), up to 120 °C. Glycerol is a water-soluble and physiologically acceptable chemical with the ability to increase the stability of lipid vesicles and does not need to be removed from the final liposomal product. Temperature and mechanical stirring provide adequate energy for the formation of stable liposomes.

Reza Mozafari et al. confirmed by TLC that no degradation of the used lipids occurred at the above mentioned temperatures. The particle size can be controlled by the phospholipid nature and charge, the speed of the stirring and the shape of the reaction vessel. Otherwise, employment of heat abolishes the need to carry out any further sterilisation procedure reducing the time and cost of liposome production.

3.2.2.2. Spray-Drying. Since spray-drying is a very simple and industrially applicable method, the direct spray-drying of a mixture of lipid and drug was applied in the preparation of liposomes. The spray-drying process is considered to be a fast single-step procedure applied in the nanoparticles formulation.

Hence, liposomes were prepared by suspending lecithin and mannitol in chloroform. The mixture was sonicated for 8 min (bath sonicator) and subjected to spray-drying on a Buchi 190 M Mini Spray Dryer. The spray-drying conditions were as follows: inlet and outlet temperatures were 120 °C and 80 °C, respectively; airflow rate was 700 Nl/hr; and the flow rate was 1000 ml/hr. The dried product was hydrated with different volumes of phosphate buffered saline (PBS; pH 7.4) by stirring for 45 min. The main factor influencing the liposomal size was the volume of aqueous medium used for hydration of the spray-dried product. However, mannitol plays an important role in increasing the surface area of the lipid mixture, enabling successful hydration of the spray-dried product.

3.2.2.3. Freeze Drying. This new method was described for the preparation of sterile and pyrogen-free submicron narrow sized liposomes. It is based on the formation of a homogenous dispersion of lipids in water-soluble carrier materials. Liposome-forming lipids and water-soluble carrier materials such as sucrose were dissolved in tert-butyl alcohol/water cosolvent systems in appropriate ratios to form a clear isotropic monophase solution. Then the monophase solution was sterilized by filtration and filled into freeze-drying vials. In recent study, a laboratory freeze drier was used and freeze-drying process was as follows: freezing at −40 °C for 8 h; primary drying at −40 °C for 48 h and secondary drying at 25 °C for 10 h. The chamber pressure was maintained at 20 Pascal during the drying process. On addition of water, the lyophilized product spontaneously forms homogenous liposome preparation. After investigation of the various parameters associated with this method it is found that the lipid/carrier ratio is the key factor affecting the size and the polydispersity of the liposome preparation. Therefore, TBA/water cosolvent system was used for economy concerns.

3.2.2.4. Super Critical Reverse Phase Evaporation (SCRPE). The SCRPE is a one-step new method that has been developed for liposomes preparation using supercritical carbon dioxide. This method allowed aqueous dispersions of liposomes to be obtained through emulsion formation by introducing a given amount of water into a homogeneous mixture of supercritical carbon dioxide/L-\text{R-dipalmitoylphosphatidylcholine/ethanol under sufficient stirring and subsequent pressure reduction.}

Transmission electron microscopy observations revealed that vesicles are large unilamellar with diameters of 0.1–1.2 μm. The trapping efficiency of these liposomes indicated more than 5 times higher values for the water-soluble solute compared to multilamellar vesicles prepared by the Bangham method. The trapping efficiency for an oil-soluble substance, the cholesterol, was about 63%. Results showed that the SCRPE is an excellent technique that permits one-step preparation of large unilamellar liposomes exhibiting a high trapping efficiency for both water-soluble and oil-soluble compounds.

3.2.2.5. Modified Ethanol Injection Method. Novel approaches based on the principle of the ethanol injection technique such as the microfluidic channel method, the crossflow-injection technique, and the membrane contactor method were recently reported for liposome production.

3.2.2.5.1. The Crossflow Injection Technique. The concept of continuous crossflow injection is a promising approach as a novel scalable liposome preparation technique for pharmaceutical application. Wagner et al. used a cross flow injection module made of two tubes welded together forming a cross. At the connecting point, the modules were adapted with an injection hole. The influencing parameters such as the lipid concentration, the injection hole diameter, the injection pressure, the buffer flow rate, and system performance were investigated. A minimum of buffer flow rate is required to affect batch homogeneity and strongly influencing parameters are lipid concentration in combination with increasing injection pressures. After exceeding the upper pressure limit of the...
linear range, where injection velocities remain constant, the vesicle batches are narrowly distributed, also when injecting higher lipid concentrations. Reproducibility and scalability data show similar results with respect to vesicle size and size distribution and demonstrate the stability and robustness of the novel continuous liposome preparation technique.49

3.2.2.5.2. Microfluidization. By using a microfluidic hydrodynamic focusing (MHF) platform, Jahn et al. generated liposomes by injecting the lipid phase and the water phase into a microchannel.45 Microfluidic flow is generally laminar due to the small channel dimensions and relatively low flow rates. Well-defined mixing is then obtained by interfacial diffusion when multiple flow streams are injected in a microchannel. The size of the liposomes was mainly controlled by changing the flow rate.44

3.2.2.5.3. Membrane Contactor. Recently, Jaafar-Maalej et al. applied the ethanol injection technique while using a membrane contactor for large scale liposomes production. In this method, a lipid phase (ethanol, phospholipid and cholesterol) was pressed through the membrane with a specified pore size. Nitrogen gas at pressure below 5 bar was sufficient for passing the organic phase through the membrane. At the same time, the aqueous phase flew tangentially to the membrane surface and swept away the formed liposomes within the membrane device. The new process advantages are the design simplicity, the control of the liposome size by tuning the process parameters and the scaling-up abilities.55

As a result, these techniques lead from the conventional batch process to potential large scale continuous procedures.

4. **IN-VITRO LIPOSOMES CHARACTERIZATION**

In order to assess the liposome quality and to obtain quantitative measures that allow comparison between different batches of liposomes, various parameters should be monitored. For liposomes applications in analytical and bioanalytical fields, the main characteristics include the average mean diameter and polydispersity index; encapsulation efficiency; the ratio of phospholipids to drug concentration and lamellarity determination. Other commonly monitored parameters include surface charge through zeta potential measurement, phase transitions through differential scanning calorimetry and quantification of residual solvents through gas chromatography. A detailed description of today’s most commonly methods and novel techniques of liposome characterization is presented in this report.

4.1. Lamellarity Determination

Lipid bilayers number of liposomes influences the encapsulation efficiency and the drugs release kinetics. Furthermore, when liposomes are taken up or processed in the cell, the intracellular fate is affected by the lamellarity. The liposomes lamellarity made from different lipids or preparation procedures varies widely. That is why, the analysis of liposomes lamellarity is an important parameter to be considered.

Liposome lamellarity is often accomplished by methods that are based on the visible or fluorescence signal change of lipids marker upon reagents addition. This approach is reviewed in more detail, since it is a relatively simple procedure that can be easily carried out in a standard lab. Several lipids can be used and results rely on the comparison of the total signal to the signal achieved from the reaction between the lipids marker and the specified reagents.58

For example the UV absorbance of 2,4,6-trinitrobenzensulfonic acid (TNBS) at 420 nm increases in the mixture as a result of complex formation with primary amines. This property has been used for the detection of aminolipids at 420 nm. As the lipid bilayers are slightly permeable to the TNBS reagent, an overestimate of the external surface can be expected. To correct the reagent leakage through the bilayer, three incubation times were used. The obtained external surface area at each incubation time was plotted against incubation time and the graph was extrapolated to time zero. Under certain conditions, the bilayer permeability of TNBS is minimized such as the only aminolipids on the exterior bilayer contribute to the signal. Lysis of liposomes by a detergent such as Triton X-100 allows TNBS to interact with interior aminolipids and yields the total signal. TNBS has remained the commonly used method for the estimation of the degree of lamellarity. However, this method has disadvantages which make it impotent in most cases; the TNBS assay requires large amount of material (milligrams) which makes the multiple sample application difficult and affect assay precision when the amount is limited.59,60

In another method, the addition of periodate to phosphatidylglycerol results in the diol oxidation and releasing of formaldehyde. The released formaldehyde reacted with chromotropic acid to yield a product which was subsequently detected at 570 nm. This method has been used for the determination of external reactive groups on liposomes.58

Otherwise, the quenching of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD) fluorescence is obtained by sodium dithionite. NBD-labeled lipids are highly fluorescent at low concentration (<1 mol%) in membranes, but undergo self-quenching at increased concentrations. In this approach, the initial NBD labelled lipids fluorescence is from all lipids in the sample. Under appropriate conditions, the addition of sodium dithionite quenches the fluorescence of only the NBD labelled existing on the outer bilayer. Fluorescence was monitored on spectrofluorometer with excitation and emission wavelengths of 450 nm and 530 nm respectively. The percentage of external lipid is found by dividing the change in fluorescence upon dithionite addition by the total fluorescence.61-63
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<th>Solvent/ adjuvants</th>
<th>Size</th>
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<td></td>
<td>Cholesterol (20% w/w PL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>98%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other techniques for lamellarity</td>
<td>Idem</td>
<td></td>
<td>Ethanol or ethanol/tert-butanol (1:1 v/v)</td>
<td>200 nm</td>
<td>300 nm</td>
<td>25%</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td>determination include small angle X-ray</td>
<td>Idem</td>
<td></td>
<td>Ethanol or ethanol/tert-butanol (1:1 v/v)</td>
<td>200 nm</td>
<td>300 nm</td>
<td>25%</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td>scattering (SAXS). For this purpose,</td>
<td>Idem</td>
<td></td>
<td>Ethanol or ethanol/tert-butanol (1:1 v/v)</td>
<td>200 nm</td>
<td>300 nm</td>
<td>25%</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td>liposome dispersions put into glass</td>
<td>Idem</td>
<td></td>
<td>Ethanol or ethanol/tert-butanol (1:1 v/v)</td>
<td>200 nm</td>
<td>300 nm</td>
<td>25%</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td>capillaries and curves were recorded with</td>
<td>Idem</td>
<td></td>
<td>Ethanol or ethanol/tert-butanol (1:1 v/v)</td>
<td>200 nm</td>
<td>300 nm</td>
<td>25%</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td>a camera equipped with a</td>
<td>Idem</td>
<td></td>
<td>Ethanol or ethanol/tert-butanol (1:1 v/v)</td>
<td>200 nm</td>
<td>300 nm</td>
<td>25%</td>
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<td>(47)</td>
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</table>

These methods appear to close a gap in the methodology to determine external surface structure of vesicles. However, these methods assume that the lipid of interest is distributed evenly over all lipid layers, and the reagents used to elicit the signal change are impermeable to the membrane over the time course of the measurements.64

Other numerous methods for the lamellarity determination such as magnetic resonance were mainly used to study the outside-inside distribution of phospholipids within bilayer and the characterization of model membrane structures. A straightforward application of nuclear magnetic resonance in the quality control of liposomes is the determination of size and lamellarity. Dispersions of MLVs give rise to very broad powder 31P-NMR spectra due to the restricted anisotropic motion whereas SUVs are characterized using a narrow line spectra. It is well known that the paramagnetic ion Mn2+ interacts with the negatively charged phospholipids phosphate causing perturbations of the nuclear spin relaxation times which broaden the 31P-NMR resonance and reduces the quantifiable signal. Presuming that the shift reagent (Mn2+) only interacts with the phospholipids located in the outermost monolayer, the degree of lamellarity can be calculated by the ratio of 31P-NMR signal before and after Mn2+ addition. Used for a long time in the field of liposome research, this technique has been found to be quite sensitive to experimental conditions which can have distinct effect on the analysis. For example, Mn2+ is able to penetrate the liposomal bilayer especially when used at high concentrations. At low pH or in the presence of complexing agents (such as HEPES or TRIS buffer at certain concentration), no penetration of Mn2+ occurs. Therefore, under well-defined conditions, the analysis of liposomes by 31P-NMR is the presence of shift reagent in an elegant and accurate method giving useful information about the outer to inner phospholipids ratio amount.65,66

Other techniques for lamellarity determination include small angle X-ray scattering (SAXS). For this purpose, liposome dispersions put into glass capillaries and curves were recorded with a camera equipped with a
one-dimensional position sensitive detector. Blank scattering curves were obtained from the same capillaries filled with the liposome suspension solvent. Data were evaluated using the Indirect Fourier Transformation which provides the electron distance distribution p(r) (the probability to find two electrons with distance r in the measured sample). SAXS is considered as a good method evaluating vesicles lamellarity with high accuracy.67

To confirm the lamellarity results by an imaging method, freeze fracture technique with subsequent transmission electron microscopy was used. For this purpose, carbon film grids were used for specimen preparation. A drop of the sample was put on the untreated coated grid. Most of the liquid was removed with blotting paper leaving a thin film stretched over the holes. The specimens were instantly shock-frozen in melting nitrogen or by plunging them into liquid ethane or propane in a temperature-controlled freezing unit. After freezing, the specimens were inserted into a cryo-transfer holder and transferred to a cryo-electron microscope. To determine the mean lamellarity, micrographs of three different areas of the specimen were investigated.66,69,70

Whatever is the technique, the lamellarity determination is essential to define liposome structure as it is a very important prerequisite for liposomes success in therapy.

4.2. Size Analysis

The average size and size distribution of liposomes are important parameters especially when the liposomes are intended for therapeutic use by inhalation or parenteral route. Several techniques are available for assessing submicrometer liposome size and size distribution which include microscopy techniques, size-exclusion chromatography (SEC), field-flow fractionation and static or dynamic light scattering.

Several variations on electron microscopy (EM) such as transmission EM using negative staining, freeze fracture TEM, and cryo EM, provide valuable information on liposome preparations since they yield a view of morphology and can resolve particles of varying size. However, sample preparation is complicated as it requires removal of liposomes from their native environment. These techniques can also generate artefacts, induce shrinkage and shape distortion, and are time consuming to obtain a representative size distribution of the population, thus are not amenable to being routine measurements. Some of these problems may be overcome yielding reproducible and accurate results by giving careful attention to sample preparation. A recently developed microscopic technique known as atomic force microscopy (AFM) has been utilized to study liposome morphology, size and stability. AFM, scanning probe microscopes with dimensional resolution approaching 0.1 nm, provides unique possibility for visualizing small liposomes in natural environment even without sample manipulation. The result is with a high resolution three-dimensional profile of the vesicle surface under study. The technique permits liposomes visualization without alteration of their native form; given that the requisite surface immobilization does not adversely affect the sample and that the force of the probe itself does not have deleterious effects on the vesicles. AFM analysis is rapid, powerful and relatively non invasive technique. It can provide information on morphology, size, as well as on the possible aggregation processes of liposomes during their storage. Imaging in aqueous medium allows the liposomes observation under physiological condition. Using AFM technology, experimental data indicate that liposomes in water dispersion maintained their integrity only few minutes after deposition on mica support, after which they collapsed. For this reason, the liposomes images have to be obtained within 10 min after deposition. Therefore, special attention has to be given to the experimental conditions and especially the analytical times, AFM technique can replace the wide variety of microscopic techniques measuring liposomal size.71–74

HPLC using SEC can be used to separate and quantify liposome populations according to a time-based resolution of hydrodynamic size. The porous packing material used in this technique excludes large species from the internal pore volume leading to their shorter retention on the column. This mechanism leads to separation based on large particles elution before smaller particles. Conventional SEC is frequently used for liposomes separation from unencapsulated materials as a final purification step, but the use of HPLC-SEC for analysis offers increased resolution of liposome populations and reduced sample size and enhances reproducibility. One recommended commercially available column is the ethylene glycol-metacrylate gel which has a separation range from 20 to 500 nm, this ‘hydroxylated poly-ether-based’ gel shows a larger exclusion limit than other gels. An osmotically balanced mobile phase flowing at relatively low pressures (1–1.5 mega-pascal) helps to prevent damages, swelling or shrinkage of liposomes. HPLC-SEC can offer a powerful technique for not only size distribution determination, but also stability in terms of aggregation and vesicle permeability. Three methods have been described in literature: dynamic light-scattering analysis of SEC fractions; rechromatography of SEC fractions on a calibrated column with turbidity measurements; and SEC with on-line turbidity and refractive index detection. The rechromatography method was judged to be the most reliable, although the sensitivity suffered from the dilution in the two chromatographic steps. Disadvantages of HPLC for liposomes size determination mainly stem from recovery issues. These include unwanted adsorption of lipids on the column packing and destruction of liposomes containing lipids with higher affinity to the column material than the composite lipids. Both lipids necessitate a preliminary step of
Field-flow fractionation (FFF) is a technique which overcomes some of the limitations of HPLC in liposome analysis. It includes electrical, thermal, sedimentation and flow FFF techniques that rely on a field application which is perpendicular to the direction of flow. FFF uses a channel wall which consists of a semipermeable membrane chosen with a MWCO suitable for the liposomes under study. This membrane allows only the carrier fluid to pass. In flow FFF, there are two liquids flows acting on the sample components. The channel flow that runs through the channel and the crossflow that flowing perpendicular to the channel passes through the inlet frit into this channel and exits through the membrane and outlet frit. A common procedure for sample injection is called ‘stop-flow relaxation’, in which a small volume sample is injected into the channel flow. After a short delay period that allows the sample to move into the channel from the injector, the channel flow is stopped for a time, allowing only the crossflow to act on the sample. The laminar flow profile slow down the movement of particles located closer to the channel walls, while the perpendicular flow propels all particles toward the membrane wall. Diffusion due to Brownian motion of particles in a size-based manner reduces the accumulation of smaller particles against the membrane wall. Retention times in this technique are proportional to the hydrodynamic diameter of the particles since smaller particles reach an equilibrium position further from the channel walls. Whereas in HPLC-SEC, large liposomes elute first, in normal mode FFF, small liposomes elute first due to their higher diffusion coefficient. The carrier liquid used in FFF needs to be chosen carefully so that there is no appreciable swelling of the membrane, as this can lead to non-uniform flows in the channel. Aqueous solutions are usually used as carrier liquids, although non-aqueous solvents have also been used. Many detectors have been used in FFF, but the most common is a UV/VIS spectrophotometer. Photodiode arrays have been used to obtain the entire spectra of eluting samples instead of monitoring a single wavelength. The FFF mechanism for liposomes analysis differs in that FFF flow separates vesicles on a hydrodynamic size basis, whereas sedimentation FFF separates them on a weight basis. Flow FFF enables rapid, convenient and non-invasive measurement of vesicle size distribution without prior calibration using size standards. Other advantage of the FFF technique is the wide range of particle sizes that can be separated (1 nm – 100 μm) with high resolution. The only limit of this technique is the complexity and expense of instrumentation.

Dynamic light scattering (DLS), otherwise known as photon correlation spectroscopy (PCS), is extensively used in liposome size distribution analysis. DLS measures the time-dependant fluctuations of light scattered from particles experiencing Brownian motion, which results from collisions between suspended particles and solvent molecules. When a particle is suspended in a solution and illuminated by light, it scatters light given that its index of refraction differs from that of suspending solvent. In other words, its polarizability differs from that of the solvent. This means that the arriving electric field is oscillating and is able to displace the cloud of electrons and thereby cause atoms to oscillate. The strengths of the technique include the ability to make measurements in native environments; its sensitivity to small quantities of high molecular weight aggregate; ease of commercially available operating instrument; minimal sample volume, concentration and preparation requirements. It also covers a large size range of species spanning the low nanometer to low micrometer range. However, the technique does not yield particle shape information; it can yield a bias towards reporting larger diameters when small quantities of high molecular weight or aggregates or impurities are present in the sample.

Measurement of particle size distribution could also be achieved using density gradient stabilized sedimentation whereby particles that are lower in density than the fluid in which they are suspended can be accurately analysed. Centrifugal sedimentation of particles suspended in a fluid is a well-known method of measuring the size distribution of particles in the range of 0.015–30 μm in diameter. The sedimentation velocity of any particle could be calculated if the particle density, fluid density, fluid viscosity, and centrifugal acceleration are known. If the conditions of sedimentation are stable, the particles begin sedimentation as a very thin layer at the surface of the fluid. A light beam or an X-ray beam passes through the centrifuge at some distance below the surface of the fluid and measures the concentration of particle as they settle. The time required for particles to reach the detecting beam depends upon the speed and geometry of the centrifuge, the difference in density between the particles and the fluid and the diameter of the particles. The particles sediment at velocities, depending upon their size until reaching the detector beam which is positioned at a known distance below the fluid’s surface. The most common method of measuring the size distribution is by using a technique called ‘stop-flow relaxation’. In this technique, a small volume of sample is injected into the channel flow. After a short delay period, the channel flow is stopped for a time, allowing only the crossflow to act on the sample. The laminar flow profile slows down the movement of particles located closer to the channel walls, while the perpendicular flow propels all particles toward the membrane wall. Diffusion due to Brownian motion of particles in a size-based manner reduces the accumulation of smaller particles against the membrane wall. Retention times in this technique are proportional to the hydrodynamic diameter of the particles since smaller particles reach an equilibrium position further from the channel walls. Whereas in HPLC-SEC, large liposomes elute first, in normal mode FFF, small liposomes elute first due to their higher diffusion coefficient. The carrier liquid used in FFF needs to be chosen carefully so that there is no appreciable swelling of the membrane, as this can lead to non-uniform flows in the channel. Aqueous solutions are usually used as carrier liquids, although non-aqueous solvents have also been used. Many detectors have been used in FFF, but the most common is a UV/VIS spectrophotometer. Photodiode arrays have been used to obtain the entire spectra of eluting samples instead of monitoring a single wavelength. The FFF mechanism for liposomes analysis differs in that FFF flow separates vesicles on a hydrodynamic size basis, whereas sedimentation FFF separates them on a weight basis. Flow FFF enables rapid, convenient and non-invasive measurement of vesicle size distribution without prior calibration using size standards. Other advantage of the FFF technique is the wide range of particle sizes that can be separated (1 nm – 100 μm) with high resolution. The only limit of this technique is the complexity and expense of instrumentation.
surface. Sedimentation velocity increases as the square of the particle diameter, so that particles which differ in size by only few percent settle at significantly different rates. The time needed to reach the detector is used to calculate the size of the particles. This method for size analysis has a high resolution compared to the other analysis method, it has also a high sensitivity which enables him to detect small additional peaks and pick up small changes. Moreover, high accuracy is assured since all analyses are run against a known calibration standard; the calibration can be either external (standard injected before the sample) or internal (standard mixed with the sample).

Several other techniques, considered to be less conventional, have been applied for liposome size distribution analysis but are not discussed in this paper, such as NMR, flow cytometry, capillary zone electrophoresis, etc.

4.3. Zeta Potential

Three of the fundamental states of matter are solids, liquids and gases. If one of these states is finely dispersed in another then we have a ‘colloidal system’. Most colloidal dispersions in aqueous media carry an electric charge. There are many origins of this surface charge depending upon the nature of the particle and its surrounding medium. The more important mechanisms are: ionization of surface groups (dissociation of acidic groups on the surface of a particle giving a negatively charged surface, conversely a basic surface will take on a positive charge) and adsorption of charged species (surfactant ions may be specifically adsorbed on the particle surface leading in the case of cationic surfactants to a positively charged surface and in the case of anionic surfactants to a negatively one).

The zeta potential of a particle is the overall charge that a particle acquires in a particular medium. It is a physical property which is exhibited by any particle in suspension. It has long been recognized that the zeta potential is a very good index of the interaction magnitude between colloidial particles. Measurements of zeta potential are commonly used to predict the stability of colloidal systems. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other and there will be no tendency to aggregation. However, if the particles have low zeta potential values then there will be no force to prevent the particles flocculating.

To measure the zeta potential, a laser is used to provide a light source illuminating particles within the samples. The incident laser beam passes through the centre of the sample cell and the scattered light at an angle of about 13° is detected. When an electric field is applied to the cell, any particles moving through the measurement volume will lead to fluctuation of the detected light with a frequency proportional to the particle speed. This information is passed to a digital signal processor, then to a computer and hence potential zeta is calculated. Particles suspension with zeta potentials > +30 mV or < −30 mV are normally considered stable.

4.4. Encapsulation Efficiency

The liposome preparations are a mixture of encapsulated and un-encapsulated drug fractions. The first step for the determination of the encapsulation efficiency is the separation between the encapsulated drug (within the carrier) and the free drug. Several separation techniques have been reported in the literature. The mini-column centrifugation is a method based on the difference of size between the drug loaded liposomes and the free drug. Indeed, undiluted liposome suspension is applied dropwise to the top of sephadex gel column and the column is spun at 2000 rpm for 3 min to expel the void volume containing the liposomes into the centrifuge tube. Then 250 µl of water was added and centrifugation was repeated. The non entrapped drug remained bound to the gel, while vesicles traversed the gel and were collected from the first and second stage of centrifugation.

The separation between the free drug and the encapsulated drug could also be achieved by the use of a dialysis membrane with an appropriate cut-off. The liposome sample is dialysed against a buffer solution for 2 hours.

The ultracentrifugation technique was reported as a simple and fast method for the separation of drug-loaded liposomes from their medium. The sample is centrifuged at 50000 rpm for 50 min at +4 °C. Centrifugation at 3000 rpm for 30 minutes can also be used. But prior to the centrifugation, liposomes should be aggregated in order to enable their sedimentation by adding an equal volume of protamine solution (10 mg/ml) to the sample.

Once drug-loaded liposomes are separated from their medium, the lipidic bilayer is disrupted with methanol or Triton X-100 and the released material is then quantified. Techniques used for this quantification depend on the nature of the encapsulant and include spectrophotometry, fluorescence spectroscopy, enzyme-based methods and electrochemical techniques.

Other methods such as HPLC or FFF can also be applied for the determination of the encapsulation efficiency. In this case, the encapsulation percent can be expressed as the ratio of the un-encapsulated peak area to that of a reference standard at the same initial concentration. This method can be applied if the liposomes do not undergo any purification (SEC, dialysis…) following preparation. Either technique are applied to separate liposome encapsulating materials from un-encapsulated drug and hence can also be used to monitor the storage stability in terms of leakage or the effect of various disruptive conditions on the retention of encapsulants. In some cases, size distribution and encapsulation efficiency determinations could be combined in one assay by using FFF coupled to a concentration detector suitable for the encapsulant.
The terminology varies widely with respect to the ability of various lipidosome formulations to encapsulate the target molecules. Many papers express results in term of ‘percent encapsulation’, ‘incorporation efficiency’, ‘trapping efficiency’ or ‘encapsulation efficiency (EE)’ which is typically defined as the total amount of encapsulant found in the liposome solution versus the total initial input of encapsulant solution. This value depends not only on the ability of the liposomes to capture the encapsulant molecules (dependent on lipid/buffer composition, liposome lamellarity, preparation procedure…) but also on the initial molar amount of encapsulant.95

Other authors define the encapsulation efficiency, or encapsulation capacity, as the molar amount of marker per mole of lipid which is obtained by dividing the concentration of encapsulant by the concentration of lipid. A similar definition is suggested expressing EE on a weight (mg) encapsulant per mM of lipid basis.22 Another commonly used parameter is the captured volume, defined as μL of entrapped volume/μmol of lipid. This number ranges from 0.5 μL/μmol for SUV and MLV to 30 μL/μmol for LUV. Unlike the ‘percent encapsulation’ parameter cited previously, these representations require knowledge of the phospholipid concentration.94, 95

4.5. Lipid Analysis

Several chemistry techniques are commonly used for the determination of phospholipid content. Most of these techniques include the use of molybdate-containing reagents yielding a blue-colored product. One such method is the Bartlett assay which relies on the digestion of organic materials in liposome samples by 160 °C sulfuric acid, oxidation to inorganic phosphates by hydrogen peroxide, phosphomolybdate formation upon interaction with ammonium molybdate, followed by reduction through interaction with 1,2,6-aminonaphtolsulfonic acid at 100 °C. A blue product is formed which can then be analysed at 830 nm for the quantitative assessment of the phospholipids in the preparation.96

In the ascorbic acid method, ammonium molybdate reacts with orthophosphates formed from acid digestion to yield phosphomolybdic acid. This compound is then reduced with ascorbic acid to yield phosphomolybdic acid. This compound is then reduced with ascorbic acid to yield a blue-colored solution, which is then oxidized to yield quinoneimine dye which is quantified at 505 nm.97

Enzymatic assays for phosphatidylcholine and cholesterol analysis are commercially available and widely used. The former method used phospholipase D to hydrolyze phospholipids and release free choline. The free choline is then oxidized to form betaine aldehyde, betaine and hydrogen peroxide, by choline oxidase. The generated hydrogen peroxide causes oxidative coupling of phenol and 4-aminoantipyrine mediated by peroxidase to yield quinoneimine dye which is quantified at 505 nm.98

The latter method relies on hydrolysis of cholesterol esters with cholesterol ester hydrolase, followed by oxidation of the cholesterol by cholesterol oxidase and subsequent production of hydrogen peroxide. This product also oxidatively couples 4-aminoantipyrine to phenol in the presence of peroxidase to yield a blue-colored quinoneimine dye which shows strong absorption at 505 nm.99, 98

Chromatographic techniques such as HPLC, GC and thin layer chromatography (TLC) can be used to separate and quantify the lipids composing lipid bilayers.100 Chromatographic approaches are advantageous since they can separate and quantify each lipid in the mixture. TLC methods for phospholipid analysis often rely on lipid separation using a mixture of chloroform, methanol and water. Detection is frequently accomplished using molybdenum blue in sulfuric acid and ninhydrin stains for the detection of phosphate and primary amino groups, respectively. For HPLC analysis, detection of lipids in the UV range is limited to 200–210 nm due to their lack of chromophores. GC analysis of lipids typically requires a derivatization step to ensure sufficient volatility of the components, either through trimethyl silylation or methyl esterification prior to detection by flame ionization or mass spectroscopy. In many cases, pre-treatment of liposomes to disrupt the lipid bilayers is completed prior to chromatographic analysis including dilution of the liposome suspension with alcohols such as 2-propanol, ethanol or methanol. The procedure choice is dependent on the mobile phase and the degree of lipid solubility.

4.6. In-Vitro Drug Release

In vitro drug release can be performed using the dialysis tube diffusion technique. The dialysis bag membrane should be selected following screening of various membrane, no drug adsorption may occur and the membrane should be freely permeable to the active ingredient (the cut off molecular weight shouldn’t be a limiting step in the diffusion process). Some millilitres aliquot of liposome suspension is placed in the dialysis bag, hermetically tied and dropped in the receptor compartment containing the dissolution medium. The entire system is kept at 37 °C under continuous magnetic stirring and the receptor medium is closed to avoid evaporation of the dissolution medium. The kinetic experiments are carried out respecting the sink
conditions in the receptor compartment. Samples of the dialysate are taken at various time intervals and assayed for the drug by HPLC, spectrophotometer or any other convenient method. The sample volume is replaced with fresh dissolution medium so as the volume of the receptor compartment remains constant. Every kinetic experiment is performed in triplicate and the average values are taken to establish the release profile of the drug from the liposome suspension.\textsuperscript{101, 102}

4.7. Liposomes Stability

The liposomes stability is a major consideration for liposome production and administration steps: from process to storage and delivery.

A stable pharmaceutical dosage form maintains its physical integrity and does not adversely influence the chemical integrity of the active ingredient during its life. Researchers are attempting to deliver low and high molecular weight drugs in a variety of polymer matrices and liposome suspensions. The successful introduction of dosage forms depends upon a well-defined stability study. In designing a stability study, physical, chemical and microbial parameters must be considered and evaluated. This wisdom is also required for the liposome dosage form. A stability study must include a section for product characterization and another section concerning the product stability during storage.

All liposome preparations are heterogeneous in size, the average size distribution of liposomes changes upon their storage. Liposomes tend to fuse and grow into bigger vesicles, which is a thermodynamically more favourable state. Fusion and breakage of liposomes on storage also poses a critical problem leading to drug leakage from the vesicles. Therefore, visual appearance and size distribution are important parameters to evaluate physical stability.

In the other hand, the major ingredient in the liposome formulations is the lipid. The liposomes lipids are derived from natural and/or synthetic phospholipid sources containing unsaturated fatty acids which are known to undergo oxidative reactions. These reactions products can cause permeability changes within liposome bilayer. In addition, interactions of drug with the phospholipid also alter the chemical stability; hence the stability profile of a drug molecule may entirely be different from its liposome preparation stability profile. Thus, it is essential to develop stability protocols evaluating the chemical integrity of the drug over a period of time.

Finally, majority of therapeutic liposome formulations are parenteral products and therefore must be sterilized to remove the microbial contamination from the product. Thus, it is important to control microbial stability of liposomal preparations.\textsuperscript{103, 104}

5. LIPOSOMES APPLICATIONS

5.1. Pharmaceutical Applications

The use of liposomes as systemic and topical drug delivery systems has attracted increasing attention. Liposomes can be formulated in liquid (suspension), solid (dry powder) or semi-solid (gel, cream) forms. \textit{In vivo}, they can be administered topically or via parenteral route.

5.1.1. Systemic Liposomal Drugs

After systemic (usually intravenous) administration, liposomes are typically recognized as foreign particles and consequently endocytosed by the mononuclear phagocytic system cells (MPS), mostly fixed Kupffer cells, in the liver and spleen. Liposomes can serve as an excellent drug-delivery vehicle to these cells. Thus, sterically stabilized liposome, which are not avidly taken up by MPS cells, have different biodistributions properties and have shown enhanced accumulation in sites of trauma, such as tumours, infections and inflammation. This accumulation is simply due to their prolonged circulation and small size; enabling them to extravasate.\textsuperscript{105}

Based on the liposome properties introduced above, several techniques of drug delivery can be envisaged:

—Liposomes can be applied to protect the entrapped drug against enzymatic degradation whilst in circulation. The lipids used in their formulation are not susceptible to enzymatic degradation; the entrapped drug is thus protected while the lipid vesicles circulate within the extracellular fluid. As an example, \(\beta\)-lactamase sensitive antibiotics such as the penicillins and cephalosporins have been encapsulated in order to be protected against the \(\beta\)-lactamase enzyme. Rowland et al. reported that liposomes offer protection in the gastrointestinal tract environment of encapsulated drug and facilitate the gastrointestinal transport of a variety of compounds.\textsuperscript{106} As clearly evidenced by Dapergolas, liposomes are candidates to be explored for oral delivery of peptides (insulin) and proteins (vaccines), which are orally degradable.\textsuperscript{107}

—Liposomes can be used for drug targeting. It has been proved that restricting the distribution of the drug to the specific target site should allow efficacy increase at low dose with attendant decrease of toxicity. Indeed, pumping a drug through the whole body is not only wasteful but, more fundamentally, increase undesirable side effects. Hence, the benefits of drug targeting include reducing drug waste, and it is possible to deliver a drug to a tissue or cell region not normally accessible to the free or untargeted drug.\textsuperscript{108} Liposomes have been widely applied in drug targeting especially in cancer treatment. Effective chemotherapy is severely limited by the toxic side effects of the drugs. Liposome encapsulation can alter the spatial and temporal distribution of the encapsulated drug molecules in the body, which may significantly reduce
**Table II.** Liposome applications in the pharmaceutical field.

<table>
<thead>
<tr>
<th>The intention of encapsulation</th>
<th>Molecule</th>
<th>Therapeutic class</th>
<th>Conclusion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protection against enzymatic degradation</td>
<td>Insulin</td>
<td>Hypoglycaemic agent</td>
<td>1.3 units of insulin entrapped in dipalmitoyl-phosphatidylcholine/cholesterol liposomes administered to normal rats decreased blood glucose level in 4 h to about 77% of those before treatment. Higher doses (4.2 and 8.4 units) extended this effect over 24 h. 1.0 units of insulin entrapped in the same liposomes had an even more pronounced effect in diabetic rats: levels of blood-glucose were reduced to 57% of pre-treatment values after 4 h.</td>
<td>(107)</td>
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<tr>
<td>Factor-VIII</td>
<td>Factor-VIII</td>
<td>Coagulation factor</td>
<td>Factor-VIII loaded liposomes were given orally to a patient with severe haemophilia A. Plasma concentration of factor-VIII rose to therapeutically effective levels. Factor-VIII activity did not rise when the free drug is orally administered.</td>
<td>(127)</td>
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<tr>
<td>Cysteamine</td>
<td>Cysteamine</td>
<td>Treatment of acute radiation syndrome “ARS”</td>
<td>Oral administration of liposome-entrapped cysteamine induces an increase in the concentration of exogenous sulphur compounds in plasma, liver and spleen. This fact can be related to a protection of cysteamine in the digestive tract.</td>
<td>(128)</td>
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<tr>
<td>Pencillins cephalosporins</td>
<td>Pencillins cephalosporins</td>
<td>Antibiotics</td>
<td>The entrapped drugs are protected against ß-lactamase enzyme while they are in circulation in the extracellular fluid.</td>
<td>(109)</td>
</tr>
<tr>
<td>Drug targeting</td>
<td>Cyclosporine</td>
<td>Immunosuppressive</td>
<td>The in vitro pharmacokinetics and renal toxicity of a liposomal formulation and the commercially available cyclosporine are compared. The apparent volume of distribution was significantly greater in liposomal formulation compared to the commercially form (13.82 ± 2.9 vs 7.67 ± 3.01 L/Kg), most likely due to the significantly prolonged biologic half-life (47.91 ± 13.15 vs 30.95 ± 8.89 h). Kidney function was assessed via the calculation of the glomerular filtration rate “GFR”, no dose-limiting nephrotoxicity was found with the liposomal formulation, suggesting a potential alternative to the toxic commercial formulation. A change in the pharmacokinetic parameters of cyclosporine due to liposomal encapsulation was observed. A peak concentration was reached in 50 min in case of liposomes compared with 225 min in case of commercially available formulation. The rate of absorption was significantly faster following the liposome administration. Generally, there was less inter-individual variation in pharmacokinetics parameters when cyclosporine was orally administered in the liposomal formulation. Thus, an oral liposomal formulation can be developed to offer the advantages of low variability and fast onset of action.</td>
<td>(129)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>Amikacin</td>
<td>Antibiotic</td>
<td>The clearance of liposomal amikacin was over 100-fold lower than the conventional amikacin clearance. Half-life in plasma was longer than that reported for other amikacin formulations. The levels in plasma remained &gt; 180 mg/ml for 6 days after the administration of the last dose and the free amikacin concentration in plasma never exceeded 17.4 ± 1 mg/ml. The low volume of distribution (45 ml/kg) indicates that the amikacin in plasma largely remained sequestered in long circulating liposomes. Less than half the amikacin was recovered in the urine, suggesting that the level of renal exposure to filtered free amikacin was reduced, possibly as a result of intracellular uptake or the metabolism of liposomal amikacin. Thus, by sequestering this antibiotic in liposomes with long circulation times, this formulation not only maintained antibiotic levels in the body for over 1 week after treatment, but also, significantly alters the disposition of amikacin within the body and therefore decreases the potentially toxic level of renal tubular exposure.</td>
<td>(131)</td>
</tr>
</tbody>
</table>
The intention of encapsulation | Molecule | Therapeutic class | Conclusion | Reference
---|---|---|---|---
Enhancement of drug solubilisation | Doxorubicin | Antineoplastic | Doxorubicin has been shown to have a 4.5-times-lower medium-pathology score for doxorubicin-induced cardiotoxicity than the free drug. In squamous cell lung carcinoma, the same drug is capable of reducing tumor burden to a significant extent. | (110)
Enhancement of drug solubilisation | Amphotericin B | Treatment of fungal systemic infections | Owing to its aqueous insolubility, amphotericin B is typically formulated into detergent micelles. However, micelles are unstable upon systemic administration, and several neuro- and nephrotoxicity limit the dose that can be administered. If the drug is formulated into liposomes, it is delivered much more efficiently to macrophages and, additionally, toxicity can be significantly reduced. | (111)
Enhancement of drug uptake | Polyvinylpyrrolidone | Blood plasma substitute | At an equal concentration of $^{125}$I-labelled polyvinylpyrrolidone, the rate of uptake of the liposome-entrapped macromolecule by the tissue was over 4-times that of the free macromolecule. | (132)
Enhancement of drug uptake | Vitamin K₁ | Prevention or treatment of hemorrhage | The effect of liposomal-associated vitamin K₁, administered orally, was investigated using rabbits with warfarin-induced hypoprothrombinaemia, and evaluated in comparison with other dosage forms of the vitamin. The coagulation recovery time of the liposomal preparation was much faster than that of the other preparations: This time was 6.2 h for the oral liposomal preparation, 13.6 h for solubilised vitamin and 19.6 h for stabilised emulsion. | (133)
Enhancement of drug uptake | Desferrioxamine | Chelating agent | Liposomal desferrioxamine given intravenously to iron-overloaded $^{59}$Fe labeled mice doubled the $^{59}$Fe excretion for a given dose of the drug. | (134)
Vaccination | Inactivated hepatitis A virus | Prevention of Hepatitis A | Purified hepatitis-A-virion capsule, viral phospholipids and envelope glycoprotein from influenza virus are mixed with phosphatidylcholine and phosphatidylethanolamine in the presence of excess of detergent. Liposomes containing antigen and some viral lipids and proteins are formed. The liposomes potentiate the immune response to the vaccine antigen. | (113)

unwanted toxic side effects and increase the efficacy of the treatment. The first step, therefore, is to determine the antigens that are produced by the tumour cells. Then to target the drug via specific receptor ligands, which may be specific antibodies for antigens produced by tumour cells. Two liposomal formulations have been approved by the US food and drug administration (FDA) and are commercially available in the USA, Europe and Japan for the treatment of Kaposi’s sarcoma. Doxil® is a formulation of doxorubicin precipitated in sterically stabilized liposomes (on the market since 1995) and DaunoXome® is daunorubicin encapsulated in small liposomes (on the market since 1990). Doxil® has been shown to have a 4.5-times-lower medium-pathology score for doxorubicin-induced cardiotoxicity than the free drug. In squamous cell lung carcinoma, the same drug is capable of reducing tumor burden to a significant extent. In order to enhance solubilisation, the amphotericin B, which is the drug of choice in the treatment of systemic fungal infections, has been widely studied for liposome encapsulation. Owing to its aqueous insolubility, amphotericin B is typically formulated into detergent micelles. But, micelles are unstable upon systemic administration, and several neuro- and nephrotoxicity limit the dose that can be administered. However in a stable colloid particle, such as liposomes, encapsulated drug is delivered much more efficiently to macrophages and, additionally, toxicity can be significantly reduced. Following this rationale, a lipid-based amphotericin B formulation is actually commercially available in the Europe and US market (respectively since 1990 and 1997): AmBisome® including amphotericin B into small liposomes. Otherwise, liposomes can also be used to enhance the drug intracellular uptake. The lipid formulation promotes...
the cellular penetration of the encapsulated drug especially antibiotics, reducing the effective dose and incidence of toxicity.

—According to the studies performed by Sullivan et al., liposomes may be useful as immunotherapeutic agents: the use of antigen-presenting liposomes may be a promising approach in the therapy of infectious diseases like HIV infection or Herpes simplex virus genital infection. A liposomal vaccine against hepatitis A has been successfully launched by the Swiss Serum Institute in 1994. Purified hepatitis-A-virion capsule, viral phospholipids and envelope glycoprotein from influenza virus are mixed with phosphatidylcholine and phosphatidylethanolamine in the presence of excess detergent forming liposomes leading to potentiate the immune response. The same company is developing vaccines for influenza, hepatitis B, diphtheria and tetanus.

—Cationic liposomes have been shown to complex (negatively charged) DNA, and such complexes were able to transfect cells in vitro, resulting in the expression of the protein encoded in the DNA plasmid in the target cells, and making liposomes useful in direct gene transfer. Obviously for gene therapy (the treatment of diseases on the molecular level by switching genes on or off), it was discovered that cationic lipid-based DNA complexes can transfect certain cells in vivo upon localized or systemic administration.

Today, enthusiasm for the systemic use of liposomal drugs is not as widespread as it was. While the long list of diseases considered candidate for systemic application of liposomal drugs has been reduced to just a few indications, the topical application of liposomal preparations has recently attracted more interest.

5.1.2. Topical Liposomal Drugs

Skin treatment applications of liposomes are based on the similarity between the lipid vesicles bilayer structure and natural membranes which includes the ability of lipid vesicles, with specific lipid composition, to alter cell membrane fluidity and to fuse with them. In the dermatological field, liposomes were initially used because of their moisturizing and restoring action.

In Schmid’s work, stratum corneum liposomes have been used in the treatment of atopic dry skin in order to restore the barrier function and to vehicle an active substance at the same time. The composition and properties of liposomes play an important role in their interaction and possible penetration within the epidermis. In addition, liposomes provide valuable raw material for the regeneration of skin by replenishing lipid molecules and moisture. Lipids are well hydrated and, even in the absence of active ingredients, humidify the skin. Often this is enough to improve skin elasticity and barrier function, which are the main causes of skin aging.

Later, the liposomes ability of enclosing many different biological materials and delivering them to the epidermal cells or even deeper cell layers was investigated. This offered new perspectives and leads to the conclusion that liposomes may be useful vehicles for topical drug delivery for varying skin diseases treatment.

Typically, conventional dosage forms, such as solution, creams, and ointments, deliver drugs in a concentration dependant manner across the stratum corneum. However, multilamellar liposomes can deliver drugs within 30 minutes to the stratum corneum, epidermis, and dermis in significantly higher concentrations than conventional preparations.

Among the great variety of candidates for liposome encapsulation, there are mainly three groups of drugs to be considered: corticoids, retinoids and local anaesthetics. Mezei et al. was the first to report increased corticosteroid concentrations in epidermis and corium combined with a reduced percutaneous absorption in an animal model. This is particularly important as far as adverse effects from extensive corticosteroid therapy are reduced. These findings were the same with human skin; Lasch investigated the effect of liposomally entrapped cortisol on human skin ex vivo; he revealed improved cortisol concentration profiles which is of real importance because cortisol is known to have no adverse effects in long-term therapy but to be often insufficient in the therapy of acute dermatoses. For this reason, higher drug concentrations will mean an improved therapeutic effect. In a clinical trial, Korting investigated the effect of betamethasone dipropionate in a liposomal preparation and in a commercial conventional preparation in patient suffering from atopic eczema. In this double-blind, randomized, paired trial the liposomal preparation, containing markedly less active substance was slightly superior in patients with atopic eczema reducing parameters of inflammation compared to the conventional preparation.

The retinoids is the second important group of drug which seems to be a promising candidate for liposomal encapsulation. One main field for the topical administration of retinoids is uncomplicated acne vulgaris. Commercial tretinoin gels shows local irritant effects and flare-up reactions at the beginning of the treatment. These characteristics, often compromising patient compliance, can be overcome by the liposomal formulation of tretinoin. Masini et al. reported a reduced irritancy in animal experiments after treatment with liposomal tretinoin, which may be explained by gradual drug release from the liposomal preparation. Schafer-Korting et al. performed a double-blind study to evaluate the efficacy and tolerability of liposomal tretinoin in patients with uncomplicated acne vulgaris. The results clearly showed that the less concentrated liposomal drug commands equal efficacy and less skin irritation compared to its commercial conventional counterparts. This equal efficacy might be attributed
Table III. Liposome-based products on the market.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Treated disease</th>
<th>Product</th>
<th>Company</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>Kaposi’s sarcoma and AIDS-related cancers.</td>
<td>DOXIL</td>
<td>Ben Venue Laboratories for Johnson and Johnson, USA</td>
<td>On the market since 1995 (USA) and 1996 (Europe)</td>
</tr>
<tr>
<td></td>
<td>Ovarian cancer and multiple myeloma.</td>
<td></td>
<td>Schering-Plough, Europe</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enzon Pharmaceuticals for Cephalon, Europe and for Sopheron Therapeutics, USA and Canada</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MYOCET</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Systemic fungal infections</td>
<td>AMBISOME</td>
<td>First, NeXstar Pharmaceuticals which was acquired by Gilead Sciences in 1999. Thus, the drug is marketed by Gilead in Europe and licensed to Astellas Pharma (formerly Fujisawa Pharmaceuticals) for marketing in the USA, and Sumitomo Pharmaceuticals in Japan.</td>
<td>On the market since 1990 (Europe) and 1997 (USA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AMBISOME</td>
<td></td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>Specific types of leukaemia (acute myeloid leukemia and acute lymphocytic leukemia)</td>
<td>DAUNOXOME</td>
<td>First, NeXstar Pharmaceuticals. Then the drug was sold to Diatos in 2006</td>
<td>On the market since 1996 (USA and Europe)</td>
</tr>
<tr>
<td>Inactivated hepatitis A virus</td>
<td>Hepatitis A</td>
<td>EPAXAL</td>
<td>Crucell Company who merged with the Swiss Serum and Vaccine Institute in 2006</td>
<td>On the Swiss market since 1994 and now in more than 40 country.</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>Anaesthesia for skin itching, burning or pain.</td>
<td>LMX4</td>
<td>Ferndale Laboratories, USA</td>
<td>On the US market since 1998</td>
</tr>
</tbody>
</table>

During the last few years, many attempts have been made to provide adequate local anaesthesia of the skin. For this, prolonged application time and high anaesthetic concentration are required. Studies performed by Gesztes et al. indicated that tetracaine encapsulated liposomes provide better local anaesthesia (low drug concentration and long anaesthesia duration) than a conventional anaesthetic cream. Similar results were obtained using other local anaesthetics such as lidocaine which is commercialized in the USA market since 1998 (ELA-Max®). Ethanol solutions of phospholipids much care is taken to remove the remaining traces of alcohol. Data presented by Touitou et al. indicated that the presence of ethanol with a relatively high concentration in systems of lipid vesicles, termed ethosomes, was reported to influence the stratum corneum penetration and permeation of drugs. Encapsulation experiments showed that ethosomes are able to entrap both hydrophilic and lipophilic drugs. These entrapment results were supported by in vitro studies on the delivery of drugs in and through the skin. Again, the ethosomal system was shown to be far superior to the control systems, both in terms of the drug concentration in the skin and the flux of the drug through the skin. Patches containing testosterone in an ethosomal system were compared in vivo in rabbits with the commercial patch Testoderm®. The results showed significantly higher testosterone blood levels from the ethosomal system.
5.2. Cosmetic Applications

The properties of liposomes can be utilized also in the delivery of ingredients in cosmetics. Liposomes offer advantages because lipids are well hydrated and can reduce the dryness of the skin which is a primary cause for ageing. Also, liposomes can supply replenish lipids and importantly linolenic acid to the skin. The first liposomal cosmetic product to appear on the market was the anti-ageing cream “Capture” launched by Christian Dior in 1986. Results indicated that ethosomes performed significantly better than the commercial drug form. For example, the average time to crusting of lesions was shorter for the ethosomal system. The permeation enhancement from ethosomes suggests a synergistic mechanism between ethanol, lipid vesicles and skin. Indeed, in comparison to liposomes, ethosomes are less rigid. Thus, the effects of ethanol which were considered to be harmful to classic liposomal formulations may provide the vesicles with soft flexible characteristics which allow them to penetrate more easily into deeper layers of skin. In another hand ethanol may disturbs the organization of the stratum corneum lipid bilayer and enhances its lipid fluidity.

Liposomes based on a natural marine lipid extract containing a highly polyunsaturated fatty acid ratio were recently introduced as Marinosomes® by Moussaux et al., for the prevention and treatment of skin diseases. Cansell et al. have reported that Marinosomes® contributed to reduce inflammation induced by croton oil by regulating PGE₂ and IL-8 production in keratinocyte cultures.

Table IV. Some liposomal cosmetic formulations currently on the market.

<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer</th>
<th>Key ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capture</td>
<td>Christian Dior</td>
<td>Liposomes in gel</td>
</tr>
<tr>
<td>Efect du Soleil</td>
<td>L’Oréal</td>
<td>Tanning agents in liposomes</td>
</tr>
<tr>
<td>Future Perfect</td>
<td>Esséde Launder</td>
<td>Vitamin E, A, ceramide, ceramide</td>
</tr>
<tr>
<td>Skin Gel</td>
<td>Nikko Chemical Co</td>
<td>Liposomes with humectant</td>
</tr>
<tr>
<td>Aquasome LA</td>
<td>Avon</td>
<td>Soothing cream for eye irritation</td>
</tr>
<tr>
<td>Eye Perfector</td>
<td>Elisabeth Arden</td>
<td>Liquid make-up</td>
</tr>
<tr>
<td>Flawless Finish</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.3. Food Applications

The majority of microencapsulation techniques currently used in the food industry are based on biopolymer matrices composed of sugars, starches, gums, proteins, synthetics, dextrin and alginates. Nevertheless, liposomes have recently begun to gain in importance in food products. Indeed, the ability of liposomes to solubilise compounds with demanding solubility properties, sequester compounds from potentially harmful medium, and release incorporated molecules in a sustained and predictable way can be used in food processing industry. Based on studies on liposomes for pharmaceutical and medical uses, food scientists have begun to utilize liposomes for controlled delivery of functional components such as proteins, enzymes, vitamins, antioxidants, and flavours. The applications are for example dairy products preparation, stabilization of food components against degradation, and delivery and enhanced efficiency of antimicrobial peptides.

The sustained release system concept can be used in various fermentation processes in which the encapsulated enzymes can greatly shorten fermentation times and improve the quality of the product. A classical example is cheese-making: after preliminary studies in which liposome systems were optimized the cheese ripening times were shortened by 30 to 50%. This means a substantial economic profit knowing that ripening times of some cheeses, such as Cheddar, are about one year during which they require well controlled conditions.

In addition to improved fermentation, liposomes were tried in the preservation of cheeses. Addition of nitrates to cheese milk to suppress the growth of spore-forming bacteria is questioned due to health concerns and natural alternatives are under study. Lysozyme is effective but quickly inactivated due to binding to casein. Liposome encapsulation can both preserve potency and increase effectiveness because liposomes become localized in the water spaces.
between the casein matrix and fat globules of curd and cheese.\textsuperscript{142} These applications of enhancing natural preservatives, including antioxidants such as vitamin E and C, will undoubtedly become very important due to recent dietary trends which tend to reduce the addition of artificial preservatives and increase portion of unsaturated fats in the diet.

In other areas of the agro-food industry, biocides encapsulated into liposomes have shown superior action due to prolonged presence of fungicides, herbicides, or pesticides at reduced damage to other life forms. Liposome surface can be made sticky so that they remain on the leaves for longer times and they do not wash into the ground.\textsuperscript{143}

6. CONCLUSION

Since they have been discovered in the 60’s by Bangham, liposomes have drawn attention of researchers. Nowadays, they always remain a topical issue; new preparation methods have been developed as well as new characterization techniques.

In the pharmaceutical field, liposomes have long been of great interest by offering a promising way for both systemic and locally acting drugs used for therapeutic applications in humans and animals. As a result of the great potential of liposomes in the area of drug delivery, several companies have been actively engaged in expansion and evaluation of liposome products. Most of them concern anticancer and antifungal drugs that, administered in their free form, are toxic or exhibit serious side-effects and their encapsulation into liposomal vesicles significantly diminishes these unwanted properties. However, there are few commercially available pharmaceutical products based on drug-in-liposome formulations. Liposome based formulation have not entered the market in great numbers because of some problems limiting their development.

Even that batch to batch reproducibility, low drug entrapment, particle size control, and short circulation half-life of vesicles seem to have been resolved, some other problems are still limiting the widespread use of liposomes, among them the stability issues, sterilization method and production of large batch sizes.

Some of the stability problems may be overcome by lyophilisation. The final product is freeze-dried liposome mixed with a suitable cryoprotectant that are particularly stable and have to be reconstituted immediately prior to administration.

Another challenge is the identification of a suitable method for sterilization of liposome formulations as phospholipids are thermolabile and sensitive substance to procedures involving the use of heat, radiation and/or chemical sterilizing agents. The alternative technique of liposome sterilization is filtration through sterile membranes (0.22 \(\mu\)m). However, this method is limited by liposome size and is not suitable for large vesicles (>0.22 \(\mu\)m).

Finally, the major challenge for liposome is the large scale production method. Pharmaceutically acceptable procedures are those that can be easily scaled to larger batch sizes and economically feasible. However, unlike the classical pharmaceutical dosage forms (tablets, capsules, suppository...) which are produced in large batch sizes, liposome based drugs even those already in the market are produced in small size batches and thus are costly for the manufacturers. Scale-up process to larger size batches is often a monumental task for the process development scientists.

However the accumulation of many novel experiences studying the practical aspects of liposomes, added to new developments in basic research, will bring the field of liposome biotechnology to the place it deserves in the future. An encouraging sign is the increasing number of clinical trials involving liposomes.

References and Notes


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