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Liposomes: A Review of Manufacturing Techniques and Targeting Strategies

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Abstract: Today, liposomes are among the most applied technologies for the encapsulation and delivery of bioactive agents and many different compounds in biological, pharmaceutical, medical and nutritional research. In this review, classification of liposomal vesicles, methods of their preparation and encapsulation, as well as their applications in food, cosmetics and pharmaceutical industries are reviewed. In addition, the main analytical approaches used to study liposome characteristics such as size, transition temperature, surface charge, fluidity, lamellarity, stability and encapsulation efficiency are presented. In the final part of the article, mechanisms of liposome targeting are discussed.

Keywords: Liposome composition, liposome production, oxidative stabilization, encapsulation efficiency, liposome targeting, release mechanisms.

INTRODUCTION

Microencapsulation is the packaging of small particles (known as active agents) within an encapsulating system (known as a capsule or shell). The contents of the capsule are isolated from the environmental stress that surrounds the capsule and can be released in a targeted site in a suitable time. There are several types of encapsulation systems, each made using different material such as polymers, surfactants, lipids or phospholipids. One of the most applied encapsulation systems is liposome, which is mainly composed of phospholipid molecules [1, 2].

Liposomes were first made synthetically in England in 1961 by Alec D. Bangham [3], who found that phospholipids combined with water form a sphere because one end of each molecule is water soluble, while the opposite end is water insoluble.

Liposomes are spherical, closed structures, composed of curved lipid bilayers, which enclose part of the surrounding solvent into their interior. The size of a liposome ranges from 20 nm up to several micrometers and they may be composed of one or several concentric or nonconcentric membranes, each with a thickness of about 4 nm [4].

The main constituents of liposomes are phospholipids, which are amphiphilic molecules containing water soluble, hydrophilic head section and a lipid-soluble, hydrophobic tail section. This property of phospholipids give liposomes unique characteristics, such as self-sealing, in aqueous media and makes them an ideal carrier system with applications in different fields including food, cosmetic, agriculture and pharmaceutics [3].

A significant advantage of liposome is that it can incorporate and release two materials with different solubiliteis simultaneously. One example for which is the incorporation of two antioxidant agents namely alpha-tocopherol (a lipid- soluble molecule) and glutathione (a water-soluble molecule) in the same lipid vesicle [5].

CLASSIFICATION OF LIPOSOMAL VESICLES

Liposomes are classified based on vesicle size, number of lamella and preparation method [5]. Liposomes that only contain a single bilayer membrane are called unilamellar vesicles (ULV).

Unilamellar vesicles can be divided into small unilamellar vesicles (SUV, less than 100nm) and large unilamellar vesicles (LUV, larger than 100nm) [6].

A multilamellar vesicle (MLV) is a liposome composed of a number of concentric lipidic bilayers. A vesicle composed of several non-concentric vesicles encapsulated within a single bilayer is known as a multivesicular vesicle (MVV).

Some of the carrier systems developed based on liposome technology are briefly introduced in the following sections [5]:

LIPOSOME-BASED CARRIER SYSTEM

- Immunoliposomes

One class of lipid vesicles designed for active targeting of the bioactive agents inside the body is known as immunoliposome [5]. This type of liposome is explained in more details in the following sections.

- Virosomes

Virosome or artificial viruses are another type of liposomal system used in active targeting and contain reconstituted viral proteins in their structure [7].

- Stealth Liposomes

During many years, researchers tried to develop carrier systems which can avoid phagocytosis and thus circulate longer in the blood. As a result of these studies, a kind of liposome so-called "Stealth particles" has emerged. Stealth liposome is made by covering the carrier surface with hydrophilic chains such as poly ethylene glycol (PEG) [8, 9].

- Transferosomes and Transdermal Bioactive Delivery

For transdermal delivery of bioactive agents, the carrier system needs to possess required deformability to be able to pass the skin layers. For this aim, transferosome has been introduced.

Transferosomes consist of phospholipids, cholesterol and additional surfactant molecules such as sodium cholate. Transferosomes are ultra deformable and squeeze through pores less than one-tenth of their diameter [5, 10].

- Archaeosomes

Archaeosomes can be defined as liposomes made from one or more of the polar ether lipids extracted from the domain *Archaea* (Archaeobacteria) [11]. Compared with liposomes, archaeosomes

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are relatively more thermostable, more resistant to oxidation and chemical and enzymatic hydrolysis. Considering that some liposome formulations have been found to retain their structural integrity with high encapsulation efficiency after treatments at sterilisation temperatures, the fact that archaeosomes are evenmore thermostable makes them ideal candidates to protect antioxidants during food processing [12, 13].

-Vesicular Phospholipid Gels

Vesicular phospholipid gels (VPGs) are highly concentrated phospholipid dispersions of semisolid consistency and vesicular morphology. VPGs can be prepared by high-pressure homogenization of high concentrations of phospholipid molecules [14]. VPGs can be useful as parenteral depot formulations. They are also useful as intermediates for liposome dispersions, especially those with drugs with high leakage rates and poor storage stabilities [5].

- Cochleates

Cochleates are small-sized and stable lipid-based carriers comprised mainly of a negatively charged lipid (e.g. phosphatidylserine) and a divalent cation such as calcium. They have a cigar-shaped multilayered structure [15, 16]. Hydrophobic, amphiphilic, negatively or positively charged molecules can be delivered by cochleates. The unique structure and properties of nanocochleates make them ideal candidates for oral and systemic delivery of antioxidants and other sensitive moieties [12].

- Nanoliposome

The nanometric versions of liposomes are known as nanoliposomes [5]. Nanosystems have many advantages over the micro systems such as longer circulation time in the blood stream without being recognized by macrophages, ease of penetration into tissues through capillaries and biological membranes, ability to be taken up by cells easily, demonstrating high therapeutic activity at the target site, sustaining the effect at the desired area over a period of days or even weeks, improve controlled release and precision targeting of the entrapped compounds to a greater extent [17]. Nanoliposomes are able to amplify the performance of bioactive agents by improving their solubility and bioavailability, *in vitro* and *in vivo* stability, as well as preventing their unwanted interactions with other molecules [18].

Nanoliposomes are employed for encapsulation and delivery of the antioxidants and they also can incorporate and deliver both vitamin E and ascorbic acid to a site of oxidation in the food system [12, 18].

APPLICATIONS OF LIPOSOMES

In order for a bioactive agent to exert its intended effect, it needs to be in physical contact with its physiological target. A possible approach to facilitate material transport into cells or target sites is the application of liposome. A significant advantage of liposome is that it can incorporate and release two materials with different solubilities simultaneously [19, 20]. Furthermore, targetability is another extremely useful characteristic of liposome. These particular properties make liposome to be useful in many applications due to its ability to increase the effectiveness of the encapsulated active agents and optimizing their dosage [2].

The unique properties of liposomes have triggered numerous applications in various fields of science and technology, from basic studies to gene and drug delivery [21]. Some of the main applications of liposomes are described below:

- Liposome Applications in the Pharmaceutical and Medical Research

Today liposomes are an important part of biological, pharmaceutical, and medical research. Liposomes not only serve as unique model membranes and nucleic acid delivery vehicles, but they also have been reported to be used as delivery systems of enzymes, various drugs, hormones and blood factors [22, 23].

Particular areas in which liposomes display therapeutic potentials are drug delivery, cancer treatment and gene therapy. They act as carriers for anticancer agents, anti-fungal drugs, antibacterials, anti-virals and certain anti-parasitics. Some of the other applications of liposomes include encapsulation of contrast agents for use in diagnostic X-ray and NMR imaging [24-27].

Because liposomes are one of the most effective carriers for the delivery of many different types of bioactive agents into cells, the applications of liposome-based formulations and products are extremely wide. Some of the new developments in this respect include applications in tumor targeting, gene and antisense therapy, DNA vaccination, immunomodulation, lung therapeutics, and cyclodextrin-controlled drug release in situ [28-30]. In immunology, antigens encapsulated in liposomes are used to generate antibodies, to mediate active and passive immunization and for many other applications (For a review see, [31]). The first report on the application of liposomes as immunological adjuvant was made 20 years ago by Allison and Gregoriadis [32] and since then, numerous studies showing the adjuvant action of liposomes have been published. Applications of liposomes as adjuvant include hepatitis B-derived polypeptides, subunit antigens from the influenza virus, adenovirus type 5 hexon, allergens, and polysaccharide-protein conjugates, to name a few. Several laboratories have studied liposomes made with detergent-extracted envelope glycoproteins from HIV-l, and synthetic peptide carrying a CTL epitope from the simian immunodeficiency virus gag protein [31]. Liposomes also can be taken by cells through different mechanisms such as endocytosis, phagocytosis, fusion and others [31].

More recently, liposomes have also been applied as contrast agents for molecular imaging [33].

- Applications of Liposomes in Food Science

Liposomes and nano-liposomes are used for improving and/or developing new taste, controlling the release of flavor, improving the food color and altering the texture of food components. They are also able to increase the absorption and bioavailability of nutraceutical and health supplements and develop food antimicrobials. In addition, lipid vesicles can be used to construct new food packaging materials with improved barrier and antimicrobial properties as well as some kinds of nanosensors for traceability and monitoring the condition of food during transport and storage [34].

The range of applications for micro- and nanoliposome in the food industry has been increasing because of the many advantages that liposome provide by protecting the active agent from the enzymatic and chemical changes, as well as temperature and ionic strength variations [2]. In addition, liposomes can be effective carriers for nutritionally valuable ingredients [35].

In the following sections, some applications of the liposomes in the food industry are explained:

One of the first reported liposome applications in food products was in cheese manufacture [36], in order to decrease the time and cost of the cheese ripening by adding the proteinases encapsulated in liposome to cheese mixes. Researchers have shown that encapsulated enzymes in liposome improve the stability and activity of enzymes and control their release time and improve the flavor of cheese and reduce the cost of production [37-39].

Studies have shown that liposome-entrapped proteinases reduce the firmness of cheddar cheeses but increase their elasticity and improve their flavor and liposome-entrapped lipase increases cheddar cheese cohesiveness and elasticity, but reduces the cheese firmness [40, 41]. They encapsulated negatively charged bacterial and fungal proteinase and lipases separately in extremely positively charged proliposome.

Lee *et al.* encapsulated the enzyme bromelain (used as a meat tenderizer) in liposomes and they also found that the stability and bioavailability of enzyme significantly increase [42].

Matsuzaki *et al.* and Rao *et al.* [43, 44] separately used liposome-entrapped β-galactosidase in order to aid the digestion of dairy foods by the lactose intolerance. They found out that liposomes can stabilize the enzyme during the storage.

Vitamins are also encapsulated in liposomes to enhance their retention. Tesoriere *et al.* encapsulated vitamin systems including vitamins A and E, and β-carotenes in liposome and they resulted that liposome able to extend the half-life of the entrapped antioxidants and facilitates their intracellular uptake [45]. Studies also showed that better bioavailability of the hydrophobic Coenzyme Q_{lo} obtained with encapsulation in a liposomal system compared with entrapment in a gelatin capsule [13].

Liposomes have also been used to increase the nutritional quality of dairy products by entrapping the vitamin D in cream and cheese [46]. These researches indicate that liposome can protect vitamins from degradation.

Ascorbic acid incorporated in liposomes exhibited a half-life of 100 days compared with a pure solution of ascorbic acid with a half-life of 18 days at 4 °C [47].

Another useful application is encapsulation of food preservatives in liposomes in order to control cheese spoilages. Lysozyme, a natural preservative derived from egg is used in food systems as a replacement for nitrite [38]. Liposome-entrapped lysozyme has the potential to prevent cheese spoilage.

Antimicrobial peptides have been extensively examined as potential biopreservatives in food technology. However, their stability in food because of proteolytic degradation and the potential interaction of the antimicrobial peptide with food components, might terminates to decrease their antimicrobial activity. The entrapment of bacteriocins into liposomes represents an alternative to overcome this problem [48].

Application of liposome - entrapped bactericide in dairy science and industry has also shown great potential [49]. Another group of researchers encapsulated the antimicrobials pediocin [50] and nicin Z [51-54] separately in liposomes and found that liposome-entrapped antimicrobials can reduce or eliminate undesirable interactions in foods and also have long-term preservative effects and can be a useful replacement for the synthetic preservatives that have undesirable side effects [55].

Another useful application of liposome is fortification of foods with ferrous sulfate and vitamin C [56], using haem liposome as a source of iron [57]. They resulted, by addition of haem liposomes to wheat flour, the fat content of flours increased and it also had a positive effect on the stability and rheological properties of the dough. Additionally, loaf volume and crumb uniformity was improved [57].

Encapsulation of enzymes in liposomes [58, 59], in order to stabilize the enzymes against food manufacture processes and preserving them for a long-time and maintain their useful effects in foods, is another application of liposome technology in food production industry.

Liposomes and nanoliposomes have also been employed for encapsulation and delivery of the antioxidant glutathione (GSH) [60-63]. The influence of α-tocopherol incorporation into liposomes and its effect on physical and chemical stability of biomembranes was studied by Nacka *et al.* [64]. Another liposomal antioxidant system containing ascorbic acid and α-tocopherol has been reported by Kirby [65]. In a more recent study, Mozafari *et al.* [66] incorporated two antioxidant agents, α-tocopherol and glutathione, in the same liposome composition, creating a bifunctional carrier system.

All the researchers reported thus far have shown that liposome entrapment of the ascorbic acid and α-tocopherol can prevent the degradation of these active agents and maximize their antioxidant effects [12].

Liposomes are also used to deliver minerals such as Ca^{+2} and Mg^{+2} into foods [13].

The benefits obtained through the application of liposome in food are, acting such as an effective controlled-release system which protects the active ingredients throughout the processes and ability to releases the ingredients where and when required [67].

- Applications of Liposomes in Cosmetics

Liposome applications in skin treatment are based on the similarity of the bilayer structure of liposome to that of the natural membranes, so depending on the lipid composition of liposome, they can alter cell membrane fluidity and deliver active drugs to the target site. Different forms of liposome preparations such as solution, creams, gels and ointments can deliver compounds across the stratum corneum [68, 69].

Liposomes based on a natural marine lipid containing a high polyunsaturated fatty acid (PUFA) ratio such as eicosapentaenoic acid (EPA, 20:5n−3) and docosahexaenoic acid (DHA, 22:6n−3) were recently introduced as Marionosomes® for the prevention and treatment of skin diseases [70]. These lipids exhibit anti-inflammatory properties *in vitro* and have variety of benefits regarding inflammatory skin disorders and metabolized by skin epidermal enzymes into anti-inflammatory and antiproliferative metabolites [71].

These types of liposomes provide valuable raw material for the regeneration, humidifying and softening of skin [70].

Liposomes can repair and accelerate the removal of pyrimidine dimmers after skin exposure to the ultraviolet radiation. Specific antioxidants in the liposome reduce the rate of formation of secondary ultraviolet-induced damages [72].

Anti-inflammatory agents, immunostimulants, and enhancers of molecular and cellular detoxification within liposomes could avoid age spots, dark circles, wrinkles, and other clinical aspects of skin aging [72].

Nutracosmetics are an emerging class of health and beauty aid products that combine the herbs and liposomes to maintain and enhance human beauty because of their beneficial properties, such as sunscreen, anti-aging, moisturizing, antioxidant, anticellulite, and antimicrobial effects [73].

LIPOSOME COMPOSITION

Calvagno *et al.* [74] showed that mean size, polydispersity index, zeta potential, loading capacity, drug release, antitumoral activity and intracellular uptake of the encapsulated drugs were influenced by liposome lipid composition and preparation methods.

Chemical components of liposomes are lipid and/or phospholipid molecules with various head groups. These components can be subjected to certain chemical manipulations, for instance to prepare liposomes with optimized physico-chemical characteristics. Different lipid compositions could modulate both technological and biopharmaceutical parameters of colloidal vesicles thus influencing the application of liposomes as carrier systems [74].

- Types of Phospholipids

Liposome features are strictly related to chemical properties of the phospholipids used for their preparation. In fact, lipids can modify biodistribution, surface charge, permeability, and release and clearance of liposomal formulations [75]. Type of the phospholipid ingredients also influences the encapsulation efficiency, toxicity and stability of liposome [17].

The hydrophilic- lipophilic balance (HLB) is a good indicator of the vesicle forming ability of any surfactant. Single-chain surfactants were found to be compatible with vesicle formation but they

possess less encapsulation efficiency in the presence of cholesterol [76, 77]. The most conventional groups of surfactant molecules are phospholipids and possess higher encapsulation efficiency. Phospholipids are amphipathic (amphiphilic) molecules, being both hydrophilic and hydrophobic. The head group of a phospholipid is hydrophilic and its fatty acid tail (acyl chain) is hydrophobic [5].

- Sterols

Sterols are important components of cell membranes and their presence in membranes causes significant changes with regard to bilayer stability, fluidity and permeability. There are some additives incorporated to liposome structure to enhance vesicular stability. Some of these additives (or moieties) improve the stability of liposomes by providing steric hindrance. In some other instances charged molecules are used to enhance the stability of the lipid vesicles by providing electrostatic repulsion [17]. One of the most applied molecules used to increase liposome stability is cholesterol. It is known that cholesterol is able to modulate the fluidity of lipid bilayers. It is usually used in most formulations to stabilize the system against the formation of aggregates by repulsive steric or electrostatic effects and modulate the fluidity of lipid bilayers [17]. Adding cholesterol to liposome reduce the permeability of the liposomal membrane to solutes. The amount of cholesterol to be used in the liposomal formulation mainly depends on the liposome application area [5]. Cholesterol has been shown to modify the order and mobility of the phospholipids in the bilayer and hence affects the bilayer fluidity [78, 79].

- Other Additives

The presence of polyethylene glycol on the surface of liposomes provides long circulating properties, improved stability, protecting the encapsulated drug against metabolic degradation /

inactivation and increased intracellular uptake of the vesicles [80].

In some liposome formulations, charged phospholipids such as dicethylphosphate (DCP) and stearyl amine (SA) have been used to produce charged vesicles.

Adding sphingomyelin exhibited reduction in water permeability and increasing the proton permeability of some kind of liposomes [81].

EFFECT OF THE BIOACTIVES NATURE ON LIPOSOME FORMATION

One of the most important factors is the influence of the nature of the encapsulated drug on vesicle formation [17]. The electrostatic attraction between charged bioactives and liposomes is a mean to increase the entrapment efficiency. Zucker *et al.* [82] found that high positive charge (>1) of the drug leads to high encapsulation efficiency and high charge increases drug's partitioning to the aqueous phase and its interaction with the counteranions inside the liposomes.

The chemical and physical structure of various drugs or active molecules can also have a profound effect on the burst effect in controlled release systems [83]. Fig. (**1**), represents different types of bioactives entrapped in a unilamellar liposome

METHODS OF LIPOSOME PREPARATION

There are a wide variety of conventional techniques that can be used to produce liposomal formulations. All methods for producing liposomes require lipids to be combined by some means with an aqueous phase [84, 85].

In the following sections, some of the most applied conventional methods of liposome production are described.
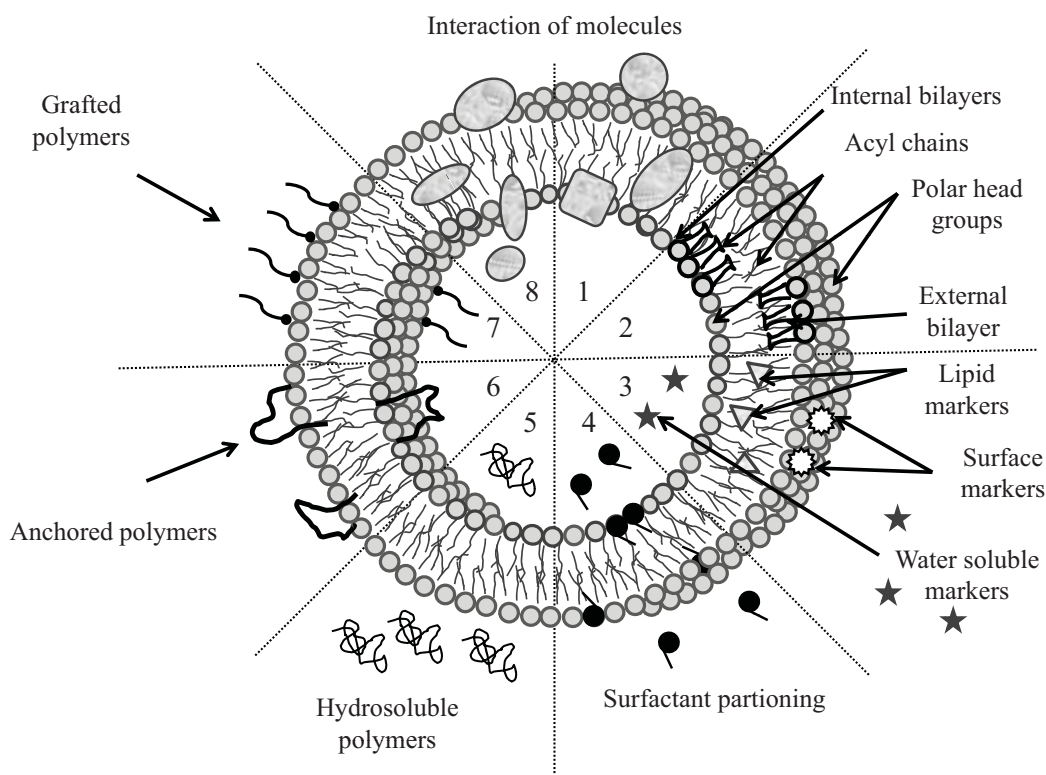

Fig. (1). Schematic representation of unilamellar liposomes (1) and positions of loaded substances (2– 8) / Hydrophobic markers are essentially located within the acyl chains of the bilayer; amphipatic labels are inserted in the polar head groups and water-soluble ones in the internal and external aqueous medium (2)/ Surfactant molecules, which are amphiphilic, partition between the liposome bilayer and the aqueous medium (3)/ Hydrosoluble polymers of various average molecular mass can be entrapped (4)/ Stealth liposomes may be formed with anchored (5) and covalently attached (6) polymers. Interaction sites of various molecules such as drugs, proteins or biological macromolecules (7, 8): water-soluble molecules can be loaded into the liposome interior or bound to the bilayer surface; amphiphilic molecules orient into bilayers; transmenbrane proteins span the lipid bilayer with sites exposed to the aqueous phase. Source: Adapted from [121].

- Bangham Method

The Bangham method is one of the first methods for liposome formation and is widely used [86, 87]. The process involves the dissolution of lipids in an organic phase, removal of the organic solvent, usually via evaporation, to form a lipid film. The solvent removal stage is time-consuming. The final step is the dispersion or hydration of the lipid film with an aqueous media, carried out in conjunction with agitation to separate the swelling lamellae from the vessel surface and form sealed spherical structure. Bangham method often produces liposomes with several microns in size and consequently MLVs which limit their consumption due to low entrapment efficiency specially for water soluble active agents, difficulty in removing organic solvent and small scale production [84].

- Detergent Depletion Method

The detergent depletion method is a mild process for the production of a wide variety of vesicle types and highly homogeneous liposomes. The method is based on the formation of detergent-lipid micelles, followed by the removal of the detergent to form liposomes [88]. The disadvantages of this method are that the final concentration of liposomes in the solution is low and entrapment of any hydrophobic compound is also low. The detergent also remains in the formulation. The size and homogeneity of liposomes produced using detergent depletion are based on the rate at which the detergent is removed and the initial ratio of detergent to phospholipid The method is very time consuming and the process of removing the detergent may also remove any other small hydrophilic compound [84, 89].

- Injection Methods

The ethanol injection method was first described in 1973 by Batzri and Korn [90]. The ethanol and ether injection methods involve the dissolution of the lipid into an organic phase, followed by the injection of the lipid solution into aqueous media, forming liposomes. The ethanol injection method is a simple method, but some lipids are poorly soluble in ethanol and heterogeneous liposomes are formed if adequate mixing is not achieved. 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 [91, 92]. Jaafar-Maalej and colleagues [93] have used this method for encapsulation the hydrophobic and hydrophilic drugs and they found that the higher encapsulation efficiencies were about 100% for the hydrophobic drug and about 16% for the hydrophilic one and they also found small multilamellar vesicles, with sizes ranging from about 80 to 170nm.

An advantage of the ether injection method compared to the ethanol injection method is to form a concentrated liposomal product with high entrapment efficiencies. The ethanol injection method is rapid, simple and reproducible for production of a ready-to-use liposome suspension. The particle size of liposomes produced by this method is a function of lipid nature and concentration, the drug to lipid ratio and the organic solvent and aqueous phase composition. The inkjet method is a modern variation of the ethanol injection method and was developed by Hauschild *et al.* [94] for liposome formation with excellent control on particle size and high potential for scaling up.

- Reverse Phase Evaporation Method

The reverse-phase evaporation process was first described by Szoka and Papahadjopoulos [95]. The technique is carried out by dissolving the lipids in an organic solvent, adding a small volume of aqueous phase, then sonicating the solution to produce inverted micelles. The organic solvent is removed using a rotary evaporator and a viscous gel forms. A disadvantage of this method is that the

compound to be encapsulated within the vesicles is in contact with an organic solvent, therefore the process is not suitable for fragile molecules such as peptides [84].

- Microfluidic Channel Method

Jahn *et al.* [96] developed a microfluidic method for controlled liposome formation. The process involves a stream of lipid dissolved in alcohol passing between two aqueous streams in a microfluidic channel, with mixing occurring at the liquid interfaces and thus liposomes forming. The laminar flow in the channels enables controlling the size and size distribution of the liposomes. Liposome self assembly by this microfluidic method can be used for drug encapsulation immediately prior to use [96].

- Heating Method

The heating method developed by Mozafari [97] to produce liposomes involves hydration of the phospholipid components in an aqueous solution containing 3% (vol) glycerol and increasing the temperature to 60°C or 120°C, depending on the absence or presence of cholesterol, respectively. Glycerol is utilized since it is a water soluble and physiologically acceptable chemical with the ability to act as an isotonising agent and increase the stability of lipid vesicles due to preventing coagulation and sedimentation. No degradation of the lipid ingredients was reported for liposomes fabricated by the heating method [98]. Also there is no need for sterilization once high temperature (i.e. 120°C) is used in this technique [5, 99]. A further improved version of the heating method, called the Mozafari method, has recently been employed for the encapsulation and targeted delivery of the food-grade antimicrobial nisin [39]. The Mozafari method allows large-scale manufacture of the carrier systems in one step without the need for the prehydration of the ingredient material, and without employing toxic solvents or detergents [39].

- Dense Gas Techniques

The term dense gas is a general expression used to refer to a substance in the region surrounding the critical point. Dense gases possess solvent characteristic similar to that of liquids along with mass transport properties similar to those of gases. The unique properties of dense gases have been exploited to replace many organic solvents and enable improved processing techniques, in particular separation, purification and size reduction processes. The most widely used dense gas is carbon dioxide since it is non-flammable, non-toxic, non-corrosive, inexpensive, environmentally acceptable and has easily accessible critical parameters (31.1°C and 73.8 bar). The solvent can be easily recovered after processing by simply returning to atmospheric pressure. Dense gas processing can provide sterile operating conditions and one-step production that can alleviate the current liposome sterilization issues [100, 101].

- Supercritical Fluid Injection and Decompression Method

The first dense gas techniques for the formation of liposomes, referred to as the injection and the decompression methods, were described by Castor and Chu in 1998 [102]. In the injection method, a mixture of lipid, organic solvent and compressed gas is injected through a nozzle into an aqueous solution. Alternatively, the decompression method involves a mixture of lipid, organic co-solvent, compressed gas and aqueous solution being decompressed through a nozzle to form liposomes. In the injection method the compressed phase is sprayed into water, whereas in the decompression method, the aqueous phase is incorporated into the compressed phase, which is sprayed into air. The injection and decompression processes are capable of producing sterile and pharmaceutical grade liposomes of a pre-determined size with narrow particle size distributions [103].

- Supercritical Fluid Method

Frederiksen *et al.* [104] described the supercritical liposome method in 1994, which is similar to the injection method developed by Castor and Chu [102] and produced small unilamellar vesicles (SUVs) with particle sizes between 20 and 50 nm [100, 104]. The process involves the dissolution of lipid and cholesterol into supercritical carbon dioxide. The solution is then rapidly expanded into an aqueous phase containing the hydrophilic compound to be entrapped. The encapsulation efficiency was lower than that achieved using conventional liposome formation techniques [105].

- Supercritical Reverse Phase Evaporation Process

Supercritical Reverse Phase Evaporation (SCRPE) method was developed by Otake *et al.* [101]. The lipid, organic co-solvent and compressed gas are combined in a stirred variable volume cell at a temperature above the lipid phase transition temperature. An aqueous solution is then slowly introduced to the cell. The pressure is reduced by the release of the compressed gas and liposomes are formed. The principle of the SCRPE method is similar to the decompression method. The trapping efficiencies achieved were extremely low [104, 105]. Otake *et al.* [106] recently developed a new method known as the improved supercritical reverse phase evaporation (ISCRPE) technique to avoid the use of organic solvents in liposome formation and enhance the stability and the drug loading efficiency of the vesicles.

- High-pressure Homogenization Method (HPH)

High–pressure homogenizers are used for the preparation of liposomes and lipid dispersions because of their vesicle disruption capability. The sample is injected at high and constant pressure in a specially designed part of the homogenizer where rearrangement of liposome structure takes place due to turbulence, cavitations and / or shear phenomena. Properties of liposomes prepared by high-pressure homogenization depend on the pressure and number of times that the sample is processed (number of cycles) [107, 108]. HPH is especially useful for the production of very small liposomes as they are especially suitable for intravenous applications [84].

- Dual Asymmetric Centrifugation (DAC)

Dual asymmetric centrifugation (DAC) is a special kind of centrifugation in which as usual a vial is turned around the main rotation axis with a defined distance and speed. The main difference of DAC to normal centrifugation is that the vial is turned around its own center (vertical axis) during the normal centrifugation process. The energy that transfers to the sample, in the form of mechanical turbulence and cavitations produces nanoliposomes with 60nm in size and homogenous size distribution. DAC has high trapping efficiency but this method is especially useful for producing batch sizes of about a gram or even less [109].

Table **1** shows the advantages and disadvantages of the conventional methods of liposome production.

TECHNIQUES FOR ENCAPSULATING BIOACTIVE AGENTS

Selection of an encapsulation protocol is largely related to parameters such as encapsulation efficiency, drug/lipid ratio, drug retention, sterility, ease of preparation and scale up, compatibility with regulatory agencies, cost efficiency, as well as liposome and drug stability [27]. Two different ways for the encapsulation of bioactive compounds in liposomes can be distinguished: (i) bioactive entrapment during the vesicle formation process (passive encapsulation) and (ii) loading the bioactive into intact vesicles (active loading) [5].

- Passive Trapping Techniques

The passive entrapment techniques rely on the ability of liposomes to capture a certain aqueous volume (including dissolved solutes) during vesicle formation [27, 110, 111]. For water soluble compounds which do not interact with the bilayer, the encapsulation efficiency after passive encapsulation is proportional to the aqueous volume enclosed by the vesicles, which itself depends on the phospholipid concentration of the dispersion and the lamellarity and morphology of the vesicles. As with the less water-soluble drugs which interact with the bilayer, both encapsulation parameters will depend more on the phospholipid concentration and selection than on morphological parameters [31]. During this process water-soluble (hydrophilic) molecules will be encapsulated inside the aqueous phase of the liposome while lipid-soluble (hydrophobic) agents will be located in the bilayer (lipidic phase) of the liposome. Amphiphilic molecules will be located in such way that the lipid-soluble part will be embedded between the liposomal lipids while their water-soluble part will be located in the liposomal aqueous phase [5].

- Active Trapping Techniques

In principle, active trapping technique consists of the blending of ''empty'' liposomes with concentrated drug solution and thereafter incubation until the drug is equally distributed by diffusion [112]. This method has some advantages, because vesicle bilayers are sufficiently permeable for drugs to allow diffusion into the liposomes within reasonable time. The drug permeates through the lipid bilayers into the vesicles following the concentration gradient until equilibrium between the interior of the vesicles and the surrounding medium is achieved [113]. The amount of hydrophobic drug that can enter in a liposome actually depends on packing restrictions in the lipid bilayer and, as a result, liposome formulations for this class of drugs change sensationally from one agent to others. Water-soluble drugs interact with the polar head groups of phospholipids and are sequestered by the liposomes but amphiphilic agents are often difficult to retain inside liposomes as they can rapidly permeate through lipid bilayers [27]. The active loading technique, however, is restricted to a small range of drugs that behave as weak amphipathic bases or acids and can permeate bilayers in the uncharged, but not in the charged, state. Active loading has certain advantages, because the active ingredient is not yet present during the preparation of the liposomes, hence the safety precautions that have to be taken when toxic drugs are handled may be minimized [114].

LIPOSOME CHARACTERIZATION

After production, characterization of liposome is required to qualify, quantify and approve the liposome capability for special application. Methods of characterization have to be exact and rapid.

-Size and Size Distribution

Calvagno *et al.* [74] have demonstrated that the mean size of liposomes was influenced by both the lipid composition and the preparation method. They found that largest mean size and polydispersity values were obtained for liposomes which were composed of oleic acid and dipalmitoylphosphatidylserine (DPPS), respectively. Oleic acid is also able to increase the liposome bilayer fluidity. Mean size and size distribution are parameters that have to be modulated as a function of the proposed application for a certain liposomal system [115].

The mean size of an aqueous dispersion of liposomes can be measured by using dynamic light scattering (DLS) operating with heterodyne detection [107, 108].

Electron Microscopy techniques (*e.g. Transmission Electron Microscopy, Scanning Electron Microscopy)* are also used as direct imaging techniques for liposomes, enabling not only quantitative analysis (the number of particles) but also qualitative information on the size and shape of liposomes. DLS provides information about the size but not about the shape of the lipid vesicles. In contrast, electron microscopic techniques [116] make direct observa-

Table 1.   Advantages and Disadvantages of Conventional Methods of Liposome Production

Methods	Advantages	Disadvantages	Application
Bangham	Simple process	Contains the organic solvent, with agitation, large vesicles without controlling on particle size, time consuming, sterilization issue [86, 87]	Diffusion of univalent ions across the liposome by Bangham *et al.* [86]
Ethanol/ether injection	Simple process	Organic solvent residue, readily to nozzle blockage in ether system, time consuming, sterilization issue [91,92]	Preparattion of Azithromycin liposomes by Wang and zhu [212]
Reverse phase evaporation	Simple design, suitable encapsulation efficiency	Not suitable for encapsulation of fragile molecule due to large quantity of organic solvent use, time consuming, sterilization issue [95]	Amphotericin B liposomes preparation by Rojanapanthu *et al.* [213]
Microfluidic channel	Control of particle size, production of vesicles with diameter up to 29 nm	Not suitable for bulk production, organic solvent use, with agitation [96]	Encapsulation of ferrous sulfate in liposome by Kosaraju *et al.* [56]
Detergent depletion	Simple design, homogenous product, control of particle size	Contains of organic solvent, detergent residue, time consuming, poor entrapment efficiency, low yield, need to sterilization [88]	Liposome preparation by Winterhalter and Lasic [214]
Supercritical fluid injection and decompression	Control of particle size, possible in situ sterilization, low organic solvent consumption	High cost, low yield, high pressure up to 350 bar used [102,103]	liposome dispersion containing an active agent by Anton *et al.* [100]
Improved/ supercritical reverse phase evaporation	No need for using nozzles, one-step production, low organic solvent consumption, rapid process, scale-up potential, enhance stability	High cost, high pressure up to 200 bar used [106]	Preparation liposome with different phospholipids by Sakai *et al.* [212]
Dense Gas Techniques	Possible in situ sterilization, producing stabilize and homogenous liposome, low organic solvent consumption	Need to multiple stages to achieve the final size of liposome, high pressure up to 200-300 bar, readily block nozzles [84]	Processing Pharmaceutical Compounds by Foster *et al.* [216]
Dual asymmetric centrifugation	Simple method, homogenous liposome production with 60 nm size, high trapping efficiency	Not suitable for bulk production, high pressure, with agitation [109]	Preparation of vesicular phospholipids which encapsulate calcein by Massing *et al.* [109]
High-pressure Homogenization method	Able to produce liposome with diameter 1oo nm, simple design, suitable for bulk production	High pressure, sterilization issue, not homogenous liposome production, organic solvent residue [107, 217]	PEG-modified CPT-11 liposomes by Li *et al.* [218]
Heating method	Simple design, organic solvent free, without need to sterilization, scalpe-up possible	Use of high temperature [97, 99]	Preparation of liposomal gene therapy vectors by Mozafari *et al.* [97]

tion possible, so the shape of liposomes and presence of any fusion or aggregation can be observed. Electron microscopic techniques also provide information on the bilayer thickness and interbilayer distance of liposome.

Atomic Force Microscopy (*AFM)* is very-high-resolution type of scanning probe microscopy that can create three-dimensional micrographs with resolution down to the nanometer and Angstrom scales [117]. AFM is also ideally suited to characterizing nanoparticles, a more recently developed microscopic technique that can be used for the structural characterization of liposomes and has also been utilized to study the morphology, size, stability, and dynamic processes of lipid nanocapsules [118, 119]. AFM allows biomolecules to be imaged not only under physiological conditions but also during biological processes.

Gel permeation chromatography was performed to compare the elution characteristics, size distribution and homogeneity of the liposomes.

Chromatography is a kind of separation method which separates molecules in solution according to their size. With organic mobile phases, the technique is known as gel-permeation chromatography and with aqueous mobile phases, the term gel-filtration chromatography has been used. The size separation takes place by repeated exchange of the solute molecules between the solvent of the mobile phase and the same solvent in the immovable liquid phase (stationary phase) within the pores of the column-packing material. The pore size range of the packing material in the column determines the molecular size range and separation can occur [5, 120].

Size Exclusion Chromatography (*SEC)* separates liposomes on the basis of size and makes it possible to estimate the molecular mass of a compound. Liposomal delivery systems can be indicated during SEC elution by separating and collecting fractions and analyzing them by *photon correlation spectroscopy* (*PCS).* By additional analyses, e.g., with an enzymatic phosphatidylcholine assay,

the lipid content of the liposomes can be determined in details [121].

-Transition Temperature (T_C)

Amphipathic molecules such as phospholipids have an important characteristic, they can undergo a thermotropic phase transition at temperatures much lower than their melting point. The temperature of transition depends on the nature of the hydrocarbon chains (acyl chain length, structure and degree of unsaturation of the hydrocarbon chains, presence of a methyl branch on the hydrocarbon chain), the polar region of the molecule and nature and ionic strength of the suspension medium (the amount of water molecules and any solutes dissolved in the water) [2, 122]. T_C, is lowered by decreased chain length, by the degree of unsaturation of the acyl chains, as well as presence of branched chains and bulky side groups. Any hydrocarbon with cis-unsaturated tail will have a lower T_C than those which are trans-unsaturated [122].

Phase transitions and fluidity of phospholipid membranes are important in the manufacture and application of liposome. The phase behavior of a liposomal membrane determines some properties such as permeability, fusion, aggregation and protein binding. All of these properties affect the stability of liposomes and their applications [5].

Transition temperature has important effects on the liposome properties. For example having low phase transition temperature is advantageous for liposomes as drug carrier systems due to the fact that actives stored in liposomes with high phase transition temperature are generally released slower than those encapsulated in liposomes with lower phase transition temperature. Consequently, determining the transition temperature of liposome is very important [69].

Differential scanning calorimetry (*DSC*) has been used extensively for the determination of transition temperatures of phospholipids [123, 124].

-Surface Charge

The surface charge of liposomes can be varied, they could be neutral (by employing phospholipids such as phosphatidylcholine, or phosphatidylethanolamine), negative (with acidic phospholipids such as phosphatidylserine, phosphatidylgelycerol, phosphatidic acid or dicetylphosphate) or positive (by the use of lipids such as dioleoyl trimethyl ammonium propane (DOTAP) or stearylamine) in physiological pH ranges. Liposomal charge is an important characteristic that determines liposome stability and encapsulation efficiency. The electrostatic attraction between charged bioactives and liposomes is a mean to increase entrapment efficiency [125, 126]. The charge density of liposomal surfaces and the binding affinity of various ions to the lipid vesicles can be determined by measuring a parameter called *zeta potential*.

-Zeta Potential

Zeta potential is a function of the surface charge of the lipid vesicle, any adsorbed layer at the interface and the nature and composition of the medium in which liposome is suspended. Zeta potential is not measurable directly but it can be calculated using theoretical models and an experimentally-determined electrophoretic mobility or dynamic electrophoretic mobility [113].

The greater zeta potential causes the liposomal suspension to be stable because the charged vesicles repel each other and thus overcome the natural tendency to aggregate. The lipid vesicles will aggregate, fuse, fluctuate and precipitate during storage. Increasing inter-particle repulsion, either electrostatic or steric, can enhance their stability [35]. The surface charge of liposomes can influence blood circulation time. Zeta potential values are influenced by lipid composition of liposomes [127].

Filion and Phillips [126] used Doppler *electrophoretic light scattering* for zeta potential measurement of liposomes formulated with cationic lipids.

Laser doppler electrophoresis (*LDE*) and *Zetasizer* [113, 124] measure the zeta potential by applying an electric field across the dispersion of liposomes.

- Fluidity

Bilayer fluidity reflects the order and dynamics of phospholipid alkyl chains in the bilayer. The influence of liposome composition on bilayer fluidity and its effect on the liposome application were investigated by Coderch *et al.* [79]. The presence of cholesterol in the membrane structure weakens Vander Waals-interactions between hydrocarbon chains of fatty acids and prevents liposome crystallization [128] and effect on bilayer fluidity. Researchers demonstrated that the incorporation of some fluid lipids into the bilayer of liposomes could interfere with the barrier function and thus lowering its phase transition temperature (T_C) and increasing its fluidity [74, 129].

The release of the entrapped drug from the liposome depends on the number of bilayers, and the bilayer permeability and fluidity of the bilayer [74, 130].

Electron paramagnetic resonance (EPR), H NMR spectroscopy and depolarization of fluorescence methods are generally used to study liposome fluidity [128].

EPR is a useful technique for determining fluidity and the structural changes of the lipid bilayers of liposomes [79].

- Lamellarity Determination

Among the most important characteristics of lipid vesicles are generally their lamellarity and size. Lamellarity is the number of lipid bilayers surrounding the inner aqueous space of the lipid vesicles. Vesicles are observed in the intermediate protonation range, as independently confirmed by various analytical techniques, for instance, electron microscopy [131].

Direct microscopical observation gives information about size, homogenity of the sample and lamellarity of liposomes [17].

Lamellarity of a liposome preparation can be also determined by using ^{31}P-*nuclear magnetic resonance* (*NMR*) to monitor the phospholipid phosphorus signal intensity at the outer monolayer compared to the total signal [132-134].

Ruozi *et al.* used *Nuclear magnetic resonance* (*NMR*) and the *Electron paramagnetic resonance* (*EPR*) to investigate the lamellarity, the permeability of the bilayer and the influence of particle size on the liposomal transport of bioactive molecules [135].

- Encapsulation Efficiency / Entrapment Efficiency

Bioactives can interact with liposomes in several different styles depending on their special properties such as solubility and polarity. They can be entrapped in the lipid bilayer phase, intercalated in the polar head groups, adsorbed on the membrane surface, anchored by a hydrophobic tail or encapsulated in the inner aqueous compartment [121].

A major achievement in the medical application of liposomes is the ability to load sufficient amount of drug needed to achieve therapeutic efficacy [82]. Liposome - encapsulated compound refers to a compound which is sequestered, at least in part, in the internal compartment of liposome or within the liposomal membrane [136].

Knowledge of liposome characteristics is required to develop liposome formulations that have optimal entrapment efficiencies and allow the controlled release of bioactives. Lipid composition and preparation method can influence the entrapping efficiency of liposome formulations. Addition of cholesterol significantly alters the entrapment efficiency [17].

The encapsulation efficiency of liposome depends on the rigidity of the bilayer membrane. Laridi *et al.* [137] encapsulated nisin Z in liposome and found that the encapsulation efficiency of 'soft' liposomes (having melting temperatures near 25°C) was about to 10%, whereas a 'hard' liposome (melting temperature of *65°C*) had an encapsulation efficiency of *35%.* They also found that by addition of fluidizing agents such as cholesterol, the encapsulation efficiency of these systems reduced [137].

Water-soluble labeled bioactive molecules (e.g. radioactive or fluorescently labeled) are used to determine encapsulation efficiency, release profile and leakage kinetics of liposomes [138].

Most of the reported experimental methods to determine liposomal encapsulation efficiency, require removal of the free (unencapsulated) bioactives from liposome encapsulated bioactives by *column chromatography* [139], size exclusion chromatography (SEC) [55], ultracentrifugation [54], equilibrium dialysis (ED)[140], ultrafilteration [141], before quantification of the entrapped material by analytical techniques such as UV/VIS Spectrometery, HPLC, Spectrofluorimetry [140, 142, 143]. Oku *et al.* [144] determined liposomal encapsulation efficiency using the fluorescent dye calcein based on fluorescence quenching of the untrapped calcein by addition of cobalt cation. This method, in contrast to the common entrapment efficiency evaluation methods, does not require application of any separation technique.

Entrapment efficiency of liposomes was determined fluorometrically by using a *fluorescence spectrophotometer* (fluoroscence quenching of calcein) by Were *et al.* [55].

Electron spin resonance (ESR) spectroscopy has also been used to determine the liposomal encapsulation efficiency whereby the addition of a paramagnetic agent such as ferricyanide results in significant broadening of the external spin-labeled marker [145]. Advantage has also been taken of the difference in diffusion coefficients between an entrapped and free marker substance such as sucrose using diffusion-ordered 2D NMR spectroscopy to assess entrapment efficiency [146].

Zhang *et al.* [139] presented a rapid and simple experimental approach using ^{1}H NMR in conjunction with a pH-sensitive marker compound (homocarnosine) to determine the liposomal encapsulation efficiency without the need to physically separate free from encapsulated marker.

-Liposome Stability

Liposome stability is one the most important factors in liposome applications and depends on a number of factors such as size and chemical composition of the vesicles. Liposomal drug products have to be stable for over two years at a minimum according to the regulatory principles of FDA [112].

Liposome is a relatively unstable colloidal system. Liposome stability can be divided into physical, chemical, colloidal and biological stability. All of these three aspects are interrelated.

The physical stability depends on the natural curvature of the lipid mixture (equilibrium curvature of the liposome), and the rigidity of the bilayer. More rigid membranes (with higher melting points) with curvatures near to their natural curvature would be more stable against disorders such as temperature increase, shear, vibration, freeze-thawing cycles [13].

Chemical stability refers to the ability of liposome to maintain the level of encapsulation efficiency with changes in solution chemistry such as pH, electrolyte composition, oxidizing agents, and presence of surface active compounds (e.g. surfactants, cholesterol, bile salts).

Colloidal stability mentioned by ability of the liposomes to maintain their size under various storage conditions [147].

Chemical degradation reduces the biological and physical stability of liposomes. Reduction of physical stability due to aggrega-

tion or drug leakage reduces liposome utility. The major chemical reactions are acyl ester bond hydrolysis and oxidative damage to polyunsaturated acyl chains, cholesterol, and (primary) amino groups. As for physical stability, the most important parameters in quality control and characterization of liposomal formulations are liposome size distribution and liposome physical integrity [21, 112].

Vesicles are stabilized based upon formation of different forces: Vander Waals forces among the phospholipids, repulsive forces among charged groups of phospholipid molecules, repulsive forces of the head groups of phospholipids and also short-acting repulsive forces.

Electrostatic repulsive forces are formed among vesicles upon addition of charged ingredients to the double layer, enhancing the stability of the system [17].

To overcome instability problems, liposomes may be freeze-dried. However, freezing may cause phase-transition changes, osmotic stress, and the expansion of bilayers due to ice formation [148]. This, in turn, may lead to bilayer disruption, fusion, and vesicle aggregation, resulting in loss of entrapped material and changes in liposome size distribution. Such effects can be minimized by the inclusion of cryoprotectants (e.g., disaccharide sugars) within the liposome formulations [149, 150]. As an alternative to freeze-drying, proliposome approach to liposome formation have been described as a mean of enhancing stability [151]. Proliposomes may be of two types: particulate-based proliposomes comprise soluble, free-flowing carrier particles coated with phospholipids [152], whilst alcohol-based proliposomes comprise a concentrated alcoholic solution of phospholipids [153]. Both these types of proliposomes generate liposomes on addition of an appropriate aqueous phase.

A) Degradative Damage upon Long-Term Storage

The long-term storage under specified conditions (e.g. temperature, light) affects the chemical and physical stability of liposomes. Some materials such as cholesterol and antioxidants provide protection against liposome degradation. Cholesterol in lipid bilayers has a role as an antioxidant in biological membranes [154]. With respect to long-term storage, two stability aspects have been considered for systems containing liposomes: (i) the liposome components may be degraded by hydrolysis and/or oxidation, and (ii) the physical structure of the liposomes may be affected, by aggregation, fusion or changes within the bilayer [112].

- *High-performance liquid chromatography* (*HPLC*) equipped with special column material with superior separation properties was introduced for the evaluation of liposome stability [155, 156]. On the detector side, several technologies such as Evaporative Light Scattering Detectors (ELSD) entered the field providing quantification approaches for liposome analysis.

- *Evaporative Light Scattering* Detectors (ELSD) is becoming the detector of choice to quantitate, UV-insensitive material such as most phospholipids. Moreover, the response of a UV detector is sensitive toward oxidative changes in phospholipids. ELSDs can be used to monitor phospholipid stability during liposome formulation studies [157].

- *Mass spectrometric* (*MS)* analysis in combination with HPLC enables quantification of different phospholipids in the sample. *Reverse-Phase High-Performance liquid Chromatography _ Electrospray ionization–Mass spectrometer* setup were able to quantify the lipids, also to assign acyl chain positions on the phospholipid molecule [158].

- Lasic and Papahadjopoulos used radiolabeled liposomes to evaluate and determine formulation parameters that would preserve physical stability under physiological conditions in circulation [31].

- Tchoreloff *et al.* used *Transmission electron microscopy* to observe the stability of liposome vesicles and their tendency to aggregate [159].

B) Lipid Peroxidation

Lipid oxidation, also called lipid peroxidation (LPO) or lipid auto-oxidation, is mediated by free radicals, leads to the formation of a broad spectrum of intermediates and products, and has long been a problem in the preparation and preservation of the lipid-based formulations [160].

In liposomal formulations, the two main types of lipid components, phospholipids and cholesterol, are susceptible to peroxidation reactions [161]. Oxidation of phospholipids takes place in their unsaturated, mainly polyunsaturated fatty acid (PUFA) chains [162].

Acyl chain and cholesterol peroxidation are interrelated. Cholesterol seems to inhibit phospholipid acyl chain peroxidation in the lipid bilayer, and the PUFA level influences cholesterol peroxidation, with higher PUFA levels decreasing cholesterol peroxidation [163, 164].

Normally, oxidation is hardly a problem in practise, but it can be minimized by using an inert atmosphere (e.g., nitrogen), metal-complexing agents (e.g., EDTA) and antioxidants (e.g., α-tocopherol) [31].

Several techniques are available for measuring and quantitating the rate of LPO in membranes. Quantitative determination of individual fatty acids (using GC) or cholesterol (using HPLC) enables an accurate follow-up of liposome lipid degradation [165].

- Variations in liposome size and polydispersity index are indicative of liposome stability. Liposome size, homogeneity, and stability can be determined by *laser light scattering*, by *gel filtration columns* and *freeze fractures Electronic Microscopy* [113].

- Oxidative Stabilization

Preventive and protective procedures are generally performed to minimize oxidative damage. Preventive procedures include the efficient chelating of ions of transition metals such as Fe^{+2} and Cu^{+2} and protection from light and from exposure to air [162].

These preventive measures are not sufficient to prevent LPO completely. Therefore, in many cases there is a need to use additional preventative methods. A common strategy of protection against LPO employs reducing agents (conventional antioxidants) that act as preventive and chain-breaking antioxidants (e.g. α- tocopherol) (Lichtenberg and Barenholz, 1988). The stabilizing effect of the various carbohydrates such as Trehalose, have also been reported [150]. In fact, a wide spreading application for Trehalose has been reported, ranging from stabilization and preservation of vaccines and liposomes to hypothermic storage of human organs [149]. Trehalose is very effective at preventing of fusion between liposomes during drying and it is able to inhibit leakage of water-soluble marker from unilamellar liposomes during freeze-drying [149, 150].

Trehalose is prominently listed as an ingredient in cosmetics [166]. Trehalose has been shown by several groups to suppress free-radical damage [167], protect against damage from anoxia [168, 169], inhibit dental caries [170], enhance ethanol stabilize and the flavors in foods [171].

C) Liposome Hydrolysis

In aqueous liposome dispersion, the liposomal phosphohpids can be hydrolyzed to free fatty acids and 2-acyl and 1-acyl lysophospholipids [172, 173]. Further hydrolysis of both of the above-mentioned lysophospholipids results in glycerophospho compounds. The hydrolysis of liposomal phospholipids is catalyzed by protons and hydroxyl ions and the hydrolysis rate reaches a minimum at pH 6.5 [174]. The effect of temperature on the hydrolysis rate of phospholipids can be adequately described by an Arrhenius equation, if no phase transitions occur in the experimental temperature range [172, 175].

- The phospholipids hydrolysis determined by quantification of Non-Esterified Fatty Acids (NEFA) by using a *Biochromatic ELISA* reader [176].

The most conventional methods for characterization assays of liposome formulation are represented in Table **2**.

LIPOSOME TARGETING MECHANISMS

One of the challenges in nanotherapy is to reduce or completely eliminate side-effects. If bioactive agents act solely on their chosen target to produce the desired effect without causing unwanted effects on other systems, their usefulness will be enhanced significantly [177]. One of the most important aspects of nanomedicine and nanotherapy is the targeting of therapeutic agents to the desired organs and tissues using special nanocarrier systems. Because of their unique properties, nanosystems enhance the performance of medicines by improving their solubility and bioavailability, increasing their stability and establishing high concentrations of bioactives in target cells and cellular compartments in order to gain therapeutic efficiency [17].

The use of liposomes for targeted drug delivery offers several advantages over direct conjugation of a targeting ligand to the therapeutic agent. First, the availability of functional groups for direct ligand conjugation to a drug molecule may be limited, rendering the conjugation chemistry problematic. Second, biological activity can be compromised, requiring the additional construction of a cleavable linker to enable drug release following endocytosis. Moreover, multiple drug molecules can be delivered upon internalization in a single liposome, whereas only single drug molecules are generally delivered following the uptake of directly conjugated agents. Thus, targeted liposomal formulations may be preferred over directly conjugated therapeutic agents [178].

Targeted delivery can be achieved by either active or passive targeting.

A) Active Targeting

Active targeting, involves the directed movement of the lipid vesicle to the given organ, tissue or cell before release of bioactive agent occurs. This can be achieved via appropriately engineered modifications to the liposomal structure. For active targeting of liposomes, thermo-labile, pH- sensitive, photo-sensitive and antibody coated vesicles have been designed [179, 180].

- Active targeting can also be achieved by utilizing external stimuli such as ultrasound, magnetic or laser field and light [181]. Experiments have shown that ultrasound focused on tumor tissue causes the release of drugs from polymeric micelles only at the tumor site [182]. This method has some advantages as well as some disadvantages. The beneficial effects, with respect to drug and gene delivery, include the loosening of cell-to-cell junctions, the permeabilization of cell membranes, the stimulation of stress response (or other) pathways in cells, the release of drugs and genes from various carriers, the deposition of heat and the activation of some chemicals by free radicals. The detrimental effects include unwanted cell injury / death and degradation of the drugs and polynucleotides. Thus one of the challenges to ultrasonic drug and gene delivery is to find the correct balance of ultrasonic parameters that maximize helpful and minimize harmful effects in order to create a functional therapeutic approach [182, 183].

- Magnetic drug targeting (MDT) uses paramagnetic particles as drug carriers, for accumulating them in target tissues by using strong local magnetic fields, and has been used in the treatment of cancer patients with some success [184]. Zheng *et al.* reported that the magnetic drug targeting was applicable in gene delivery [185].

Magnetic particles have been widely used in various aspects in biotechnology and biomedicine fields, such as biosensor, MRI

Table 2. Characterization Assays for Liposomal Formulations

Basic Assays	Methodology
pH	pH meter
Osmolarity	Osmometer
Lipid concetration	Lipid phosphorus content, HPLC, Enzymatic assays, HPTLC
Lipid composition	TLC, HPLC
Cholestrol concentration	HPLC, HOTLC, Enzymatic assays
Lipid hydrocarbon chains composition	GC
Residual organic solvent	GC
Heavy metals	NMR
Chemical stability	
Lipid hydrolysis	HPLC, HPTLC
Lipid hydrocarbon chain oxidation	Lipid peroxides, Conjugated dienes, Fatty acid composition (GC), TBA method
Free fatty acid concentration	HPLC
Cholestrol oxidation	HPLC, TLC
Physical stability	
Apperance, coclor, clarity	Pharmacopoeia methods
Vesicle size distribution, submicron range	DLS, SLS, Electron Microscopy, HPSEC, Turbidity
Micron range	Light obscuring method, Light microscopy, Laser diffraction, SLS, Coulter counter
Lamellarity	Elecctron microscopy
Zeta potential	Electrophoretic mobolity , Zetasizer
Thermotropic phase behaviour	DSC, NMR, FTIR, Fluorescence spectroscopy, Raman spectroscopy
Phase separation	DSC, NMR, FTIR, Fluorescence spectroscopy, Raman spectroscopy, ESR, turbidity, AFM
Microbiological assay	
Sterility	Pharmacopoeia methods
Endotoxin	Pharmacopoeia methods (LAL)

Abbreviations: HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer liquid chromatography; TLC, thin-layer chromatography; GC, gas chromatography; NMR, nuclear magnetic resonance; TBA, thiobarbituric acid; DLS, dynamic light scattering; SLS, static light scattering; HPSEC, high-performance size-exclusion chromatography; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared spectroscopy; ESR, electron paramagnetic spin relaxation spectroscopy; AFM, atomic force microscopy; LAL, limulus amebocyte lysate assay. Source: Adapted from [111].

contrast agent, cell separation, immunoassay, targeted drug delivery, and hyperthermia and they have a long and controlled sustained release profile by changing the number of polymeric lipid layer [186].

- Asmatulu *et al.* synthesized biodegradable magnetic nanosystems for the purpose of magnetic targeted drug delivery [187]. However, the amount of drug that magnetite nanosystems can carry is limited, and stability of the dispersion with respect of precipitation and oxidation need to be improved. Coating with polymers is a good route to solve these problems, for example, recently the results have shown that different problems are associated with drug delivery across the blood brain barrier (BBB) will be solved by using polymeric/solid lipid nanoparticles [188, 189].

A potential benefit of using magnetic nanosystems is the use of localized magnetic field gradients to attract the particles to a chosen site and the possibility to hold them until the therapy is complete [190].

- In order to release the liposomal content at the target site, photosensitivity is another property that has been introduced into liposomes. Photosensitive liposomes have been produced by conjunction of retinoids, photopolymerizable or photolabile lipids and lipoidal derivatives of retinoic acid into bilayer membranes [180]. The photoactive agents in the lipid bilayer were used for destabilizing the liposome membrane and causing the release of contents in the aqueous compartment due to exposure to UV-A, UV-B or gamma [191, 192].

- The pH-sensitive carriers destabilize endosomal membrane under the low pH inside the endosome / lysosome compartment and liberate the entrapped bioactive into the cytoplasm and the liposomes subsequently fuse with the endosomal membranes resulting in release of their contents into the cytosol [193].

- Recently, a number of methods have been developed for the modification of the liposome surface using thermally responsive polymers to enhance the temperature-sensitivity of the vesicles.

This mechanism can be used for increasing the release of anti-cancer drugs at external hyperthermic temperatures of tumors [194, 195]. Temperature-sensitive liposome is a kind of targeted delivery system, which releases its contents in response to environmental temperature. Liposomes that exhibit a response at few degrees above physiological temperature are considered to be especially useful for site-specific targeted delivery of drugs in the body because such liposomes can release drugs specifically at a target area where heat is applied [196].

Li *et al.* [197] hypothesized that: (i) The sol–gel transition temperature of thermosensitive targeted drug delivery system should be below the body temperature, and its gel–sol transition temperature should be greater than physiological body temperature , (ii) The thermosensitive drug delivery system has none or a little release in the body temperature, while it possess large accumulative release at higher temperatures of the tumor site, about (40–44°C), (iii) In order to be effective, the thermosensitivity of drug delivery systems should be among a narrow temperature range. Temperature-sensitive drug delivery system should have a reversible structural transition from a closed form to an open form as a result of changes the external temperature giving on–off switches for active drug delivery.

- Active targeting of a therapeutic agent can be obtained by conjugating the therapeutic agent or the carrier system to a tissue or cell-specific ligand. Conjugation of targeting ligands to bioactives or carriers is the most conventional method of site-specific drug delivery. For this purpose, various techniques have been devised, including covalent and non-covalent conjugation [198]. Direct coupling of bioactives to a targeting ligand restricts the coupling capacity to a few bioactive molecules. In contrast, coupling of nanocarriers to ligands allows import of thousands of bioactive molecules by means of one receptor targeted ligand [199, 200]. The coupling reactions must not affect the biological activity of ligand and should not adversely affect the structure of nanocarrier systems. Moreover, such coupling reactions must be optimized so that binding of ligands takes place in a homogeneous manner on the surface of the nanocarrier system. The identity and characteristics of the targeting moiety are important for circulation time, cellular uptake, affinity and extravasation [201]. Virosomes [202, 203], or artificial virus particles, are one type of liposomes that contain reconstituted viral proteins in their structure. Due to the presence of the specialized viral proteins on the surface of virosomes, they can be used in active targeting [177, 204].

- Another class of lipid vesicles designed for active targeting of their encapsulated / entrapped material inside the body is known as immunoliposome. The immunoliposomes possess moieties such as antibodies, carbohydrates and hormones on the outer surface of their membrane [205, 206]. Various ligands can be attached to the outer surface of the lipid vesicles by either insertion into the membrane, adsorption to the surface, via biotin - avidin pair or through the most preferable method covalent binding [161]. These ligands attached to the immunoliposome have a complementary binding site on the target cell.

B) Passive Targeting

In this method the bioactive-carrier complex reaches its destination by following the physio-anatomical conditions of the body. Site-specific delivery is achieved based on the physicochemical properties of bioactive carrier complexes and does not require utilization of any targeting strategy. The clearance kinetics and *in vivo* biodistribution of carrier systems depend on the physicochemical factors like size, charge and hydrophobicity and can be manipulated to enable passive targeting [207].

Liposomes with a mean diameter of 100-nm, for example, can selectively extravasate in tissues characterized by leaky vasculature such as solid tumors [208], while liposomes with larger diameters (≥ 1 micrometer) are taken up by the reticuloendothelial system (RES) in a passive manner. Stealth carriers are a kind of liposomes, which can be made by covering the surface of the lipid vesicles with hydrophilic chains to prevent opsonization [209]. This will provide liposomes with long circulation time and less elimination from the blood and, as a result, higher drug uptake. The vesicles will migrate and accumulate in the tumorous or infected area and as the stealth liposomes become degraded, they will release their drugs into the surrounding area [210, 211]. This is an example of passive targeting because the stealth liposomes migrate and treat the injured area passively, without employing any active targeting strategy.

SUMMARY

Due to their unique properties, including low cytotoxicity, good biocompatibility and biodegradability, liposomes possess wide applications in different fields including gene and drug delivery, food and nutrition industries and cosmetics. Most of the liposome producers are interested in using a low cost and simple process without contamination of the product. Many processes are under development to produce sterile and homogenous liposome with low cost and high stability and eliminate organic solvent usage. The method of preparation has significant effects on liposome properties such as size, shape and lamellarity, which, in turn, determine the encapsulation and entrapment efficiency. Several techniques are available for assessing physical and chemical properties of liposomes. However some of them are time consuming and involve high operation costs.

One of the most important aspects of nanomedicine and nanotherapy is the targeting of therapeutic agents to the desired organs and tissues using special nanocarrier systems of different natures to cure human diseases. Recently, a number of methods have been developed in order to modify the liposome structure and improve their stability as well as establishing high concentrations of bioactives in the target cells and cellular compartments in order to gain maximum therapeutic efficiency. Active targeting can be achieved via appropriately engineered modifications to the liposomal structure. For active targeting of liposomes, thermo-labile, pH-sensitive, photo-sensitive and antibody coated vesicles, have been designed. By passive targeting, the bioactive-carrier complex reaches its destination based on the physicochemical properties of bioactive carrier complexes and does not require utilization of any targeting strategy.

ABBREVIATIONS

AFM	=	Atomic Force Microscopy
BBB	=	Blood brain barrier
DAC	=	Dual asymmetric centrifugation
DCP	=	Dicethylphosphate
DHA	=	Docosahexaenoic acid
DLS	=	Dynamic light scattering
DOTAP	=	Dioleoyl trimethyl ammonium propane
DPPS	=	Dipalmitoylphosphatidylserine
DSC	=	Differential scanning calorimetry
ED	=	Equilibrium dialysis
ELSD	=	Evaporative Light Scattering Detectors
EPA	=	Eicosapentaenoic acid
EPR	=	Electron Paramagnetic Resonance
ESR	=	Electron spin resonance
HLB	=	hydrophilic- lipophilic balance
HPH	=	High-pressure Homogenization
HPLC	=	High-performance liquid chromatography
ISCRPE	=	Improved supercritical reverse phase evaporation

LDE	=	Laser doppler electrophoresis
LPO	=	Lipid peroxidation
MDT	=	Magnetic drug targeting
MLV	=	Multilamellar vesicle
MS	=	Mass spectrometric
MVV	=	Multivesicular vesicle
NEFA	=	Non-Esterified Fatty Acids
NMR	=	Nuclear magnetic resonance
PCS	=	Photon correlation spectroscopy
PEG	=	Poly ethylene glycol
PUFA	=	Polyunsaturated fatty acid
RES	=	Reticuloendothelial System
SA	=	Stearyl amine
SCRPE	=	Supercritical Reverse Phase Evaporation
SEC	=	Size Exclusion Chromatography
SUV	=	Small unilamellar vesicles
Tc	=	Transition temperature
ULV	=	Uunilamellar vesicles.
VPGs	=	Vesicular phospholipid gels

REFERENCES

[1]   Augustin, M.A.; Hemar, Y. Nano- and micro-structured assemblies for encapsulation of food ingredients. *Chem. Soc. Rev*., **2009**, *38*(4), 902-912.

[2]   Mozafari, M.R, Johnson, C., Hatziantoniou, S., Demetzos, C. Nanoliposomes and their applications in food nanotechnology. *J. Liposome Res*., **2008**, *18*(4), 309-327.

[3]   Bangham, A.D. A correlation between surface charge and coagulant action of phospholipids. *Nature*, **1961**, *192*(4808), 1197-1198.

[4]   Harendra, S.; Vipulanandan, C. Production and Characterization of Liposome Systems for Pharmaceutical Applications. Vipulanandan Center for Innovative Grouting Material and Technology (CIGMAT), Department of Civil and Environmental Engineering University of Houston: Houston, Texas, 77204-4003, **2006**.

[5]   Mozafari, M.R.; Mortazavi M.S. *Nanoliposomes: From Fundamentals to Recent Developments*; Trafford Publishing Ltd: UK, **2005**.

[6]   Taylor, T.M.; Davidson P.M.; Bruce B.D.; Weiss J. Liposomal nanocapsules in food science and agriculture. *Crit. Rev. Food Sci. Nutr.,* **2005**, *45*(7-8), 587-605.

[7]   Lasic, D.D. Kinetic and thermodynamic effects in the formation of amphiphilic colloidal particles. *J. Liposome Res.,* **1993**, *3*, 257-273.

[8]   Gref, R.; Minamitake, Y.; Peracchia, M.T.; Trubetskoy, V.; Torchilin, V.; Langer, R. Biodegradable long-circulating polymeric nanospheres. *Sci. Cult*., **1994**, *263*, 1600-1603.

[9]   Metselaar, J.M.; Bruin, P.; De Boer, L.W.T.; De Vringer, T.; Snel, C.; Oussoren, C.; Wauben, M.H.M.; Crommelin, D.J.A.; Storm, G.; Hennink, W.E. A novel family of L-amino acid-based biodegradable polymer-lipid conjugates for the development of long-circulating liposomes with effective drug-targeting capacity. *Bioconjug. Chem*., **2003**, *14*(6), 1156-1164.

[10]   Cevc, G. Transfersomes, liposomes and other lipid suspensions on the skin: Permeation enhancement, vesicle penetration, and transdermal drug delivery. *Crit. Rev. Ther. Drug Carrier. Syst*., **1996**, *13*(3-4), 257-388.

[11]   Krieg, A.M. From bugs to drugs: Therapeutic immunomodulation with oligodeoxynucleotides containing CpG sequences from bacterial DNA. *Antisense Nucleic Acid Drug Dev*., **2001**, *11*(3), 181-188.

[12]   Mozafari, M.R.; Flanagan, J.; Matia-Merino, L.; Awati, A.; Omri, A.; Suntres, Z.E.; Singh, H. Recent trends in the lipid-based nanoencapsulation of antioxidants and their role in foods. *J. Sci. Food Agric*., **2006**, *86*(13), 2038-2045.

[13]   Garti, N. Delivery and controlled release of bioactives in foods and nutraceuticals; Woodhead Publishing Limited: USA, **2008**.

[14]   Tardi, C.; Drechsler, M.; Bauer, K.H.; Brandl, M. Steam sterilisation of vesicular phospholipid gels. *Inter. J. Pharm*., **2001**, *217*(1-2), 161-172.

[15]   Zarif, L.; Graybill, J.R.; Perlin, D.; Mannino, R.J. Cochleates: New lipid-based drug delivery system. *J. Liposome Res*., **2000**, *10*(4), 523-538.

[16]   Zarif L. Elongated supramolecular assemblies in drug delivery. *J. Control. Release*, **2002**, *81*(1-2), 7-23.

[17]   Mozafari, M.R. *Nanomaterials and Nanosystems for Biomedical Applications*; Springer: The Netherlands, **2007**.

[18]   Mozafari, M.R. In: *Methods in molecular biology*; Clifton, N.J. Ed.; Humana press: **2010**; Vol. *605*, pp. 29-50.

[19]   Boddy, A.; Aarons, L.; Petrak, K. Efficiency of drug targeting: steady-state considerations using a three-compartment model. *Pharm. Res.,* **1989**, *6*(5), 367-372.

[20]   Stella, V.J.; Himmelstein, K.J. Prodrugs and site-specific drug delivery. *J. Med. Chem*., **1980**, *23*(12), 1276-1282.

[21]   Samuni, A.M.; Barenholz, Y. In: *Liposomes, Methods in Enzymology;* Düzgüneş N, Ed.; Elsevier Academic Press: USA, **2004**; pp. 299-314.

[22]   Shah, S.P.; Misra, A. Liposomal amikacin dry powder inhaler: effect of fines on *in vitro* performance. *AAPS Pharm.Sci.Tech.,* [electronic resource]. **2004**, *5*(4).

[23]   Müller, T.; Selhorst, T.; Schuster, P.; Vos, A.; Wenzel, U.; Neubert, A. Kinetics of maternal immunity against rabies in fox cubs (Vulpes vulpes). *BMC Infect. Dis*., **2002**, *2*, 11-16.

[24]   Lopez Berestein, G.; Mehta, R.; Hopfer, R.L. Treatment and prophylaxis of disseminated infection due to Candida albicans in mice with liposome-encapsulated amphotericin B. *J. Infect. Dis.,* **1983**, *147*(5), 939-945.

[25]   Lopez-Berestein, G.; Fainstein, V.; Hopfer, R. Liposomal amphotericin B for the treatment of systemic fungal infections in patients with cancer: A preliminary study. *J. Infect. Dis.,* **1985**, *151*(4), 704-710.

[26]   Fountain, M.W.; Dees, C.; Schultz, R.D. Enhanced intracellular killing of Staphylococcus aureus by canine monocytes treated with liposomes containing amikacin, gentamicin, kanamycin, and tobramycin. *Curr. Microbiol.,* **1981**, *6*(6), 373-376.

[27]   Mayer, L.D.; Bally, M.B.; Hope, M.J.; Cullis, P.R. Techniques for encapsulating bioactive agents into liposomes. *Chem. Phys. Lipids*., **1986**, *40*(2-4), 333-345.

[28]   Bodor, A.; Kutas, J. Liposomes in gene therapy. *Acta. Pharm. Hung*., **2003**, *73*(2), 115-122.

[29]   Manosroi, A.; Thathang, K.; Werner, R.G.; Schubert, R.; Peschka-Süss, R.; Manosroi, J. Development of highly stable and low toxic cationic liposomes for gene therapy. *Arzneim-Forsch.,* **2008**, *58*(10), 485-492.

[30]   North, S.; Butts, C. Vaccination with BLP25 liposome vaccine to treat non-small cell lung and prostate cancers. *Expert. Re. Vaccin.,* **2005**, *4*(3), 249-257.

[31]   Lasic, D.D.; Papahadjopoulos, D. *Medical application of liposomes.* Elsevier Academic Press: USA, **1998**.

[32]   Allison, A.C.; Gregoriadis, G. Liposomes as immunological adjuvants. *Nature*., **1974**, *252*, 252-255.

[33]   Reulen, S.W.A.; Brusselaars, W.W.T.; Langereis, S.; Mulder, W.J.M.; Breurken, M.; Merkx, M. Protein-liposome conjugates using cysteine-lipids and native chemical ligation. *Bioconjug. Chem*., **2007**, *18*, 590-596.

[34]   Chaudhry, Q.; Scotter, M., Blackburn, J.; Ross, B.; Boxall, A.; Castle L.; Aitken, R.; Watkins, R. Applications and implications of nanotechnologies for the food sector. *Food Addit. Contam.,* **2008**, 25, 241-258.

[35]   Keller, B.C. Liposomes in nutrition. *Trend. Food Sci. Tech*., **2001**, *12*(1), 25-31.

[36]   Law, B.A.; King, J.S. Use of liposomes for proteinase addition to Cheddar cheese. *J. Dairy Res*., **1985**, *52*, 183-188.

[37]   Magee, JR, E.L.; Olson N.F. Microencapsulation of cheese ripening systems: Stability of microcapsules. *J. Dairy Sci,*. **1981**, *64*, 611-615.

[38]   Thompson, A. Liposomes: From concept to application. *Food New Zealand*. **2003**, FEB-MARCH, S23-S32.

[39]   Colas, J.C.; Shi, W.; Rao, V.S.N.M.; Omri, A.; Mozafari, M.R.; Singh, H. Microscopical investigations of nisin-loaded nanoliposomes prepared by Mozafari method and their bacterial targeting. *Micron*., **2007**, *38*(8), 841-847.

[40]   Kheadr, E.E.; Vuillemard, J.C.; El-Deeb, S.A. Acceleration of cheddar cheese lipolysis by using liposome-entrapped lipases. *J. Food Sci.,* **2002**, *67*(2), 485-492.

[41]   Kheadr, E.E.; Vuillemard, J.C.; El Deeb, S.A. Accelerated Cheddar cheese ripening with encapsulated proteinases. *Int. J. Food Sci. Technol*., **2000**, *35*(5), 483-495.

[42]   Lee ,D.; Jin B.; Hwang, Y., Seung-Cheol-Lee, S. Encapsulation of bromelain in liposome. *J. Food Sci. Nutr.,* **2000**, *5*, 81-85.

[43]   Matsuzaki, M.; McCafferty, F.; Karel, M. The effect of cholesterol content of phospholipid vesicles on the encapsulation and acid resistance of Î²-galactosidase from *E. coli. Inter. J. Food Sci. Technol.,* **1989**, *24*, 451-460.

[44]   Rao, D.R.; Chawan, C.B.; Veeramachaneni, R. Liposomal encapsulation of beta-galactosidase: Comparison of two methods of encapsulation and *in vitro* lactose digestibility. *J. Food Biochem*., **1995**, *18*(4), 239-251.

[45]   Tesorierea, L.; Bongiornob, A.; Pintaudia, A.M.; D'Annaa, R.; D'Arpaa, D.; Livreaa M.A. Synergistic Interactions between Vitamin A and Vitamin E

against Lipid Peroxidation in Phosphatidylcholine Liposomes. *Acta Biochem. Biophys.,* **1996**, *326*(1), 57-63.

[46] Banville, C.; Vuillemard, J.C.; Lacroix, C. Comparison of different methods for fortifying Cheddar cheese with vitamin D. *Inter. Dairy J.*, **2000**, *10*(5-6), 375-382.

[47] Schrooyen, P.M.M.; Van der Meer, R.; De Kruif, C.G. Microencapsulation: Its application in nutrition. *Proc. Nutr. Soc.,* **2001**, *60*(4), 475-479.

[48] Silva Malheiros, P. da.; Daroit, D.J.; Brandelli, A. Food applications of liposome-encapsulated antimicrobial peptides. *Trend. Food Sci. Tech.*, **2010**, *21*(6), 284-292.

[49] Kirby, C. Microencapsulation and controlled delivery of food ingredients. *Food Sci. Tech. Today*., **1991**, *5*(2), 74-78.

[50] Degnan, A.J.; Luchansky, J.B. Influence of beef tallow and muscle on the antilisterial activity of pediocin AcH and liposome-encapsulated pediocin AcH. *J. Food Prot.,* **1992**, *55*, 552-554.

[51] Benech, R.O.; Kheadr, E.E.; Lacroix, C.; Fliss, I. Impact of nisin producing culture and liposome-encapsulated nisin on ripening of Cactobacillus added-Cheddar cheese. *J. Dairy Sci.,* **2003**, *86*(6), 1895-1909.

[52] Verheul, A.; Russell, N.J.; Van 'T Hof, R.; Rombouts, F.M.; Abee T. Modifications of membrane phospholipid composition in nisin-resistant Listeria monocytogenes Scott A. *Appl. Environm. Microb.,* **1997**, *63*(9), 3451-3457.

[53] Benech, R.O.; Kheadr, E.E.; Laridi, R.; Lacroix, C.; Fliss, I. Inhibition of Listeria innocua in cheddar cheese by addition of nisin Z in liposomes or by in situ production in mixed culture. *Appl. Environm. Microb,.* **2002**, *68*(8), 3683-3690.

[54] Laridi, R.; Kheadr, E.E.; Benech, R.O.; Vuillemard, J.C.; Lacroix, C.; Fliss, I. Liposome encapsulated nisin Z: Optimization, stability and release during milk fermentation. *Inter. Dairy J.,* **2003**, *13*(4), 325-336.

[55] Were, L.M.; Bruce, B.D.; Davidson, P.M.; Weiss, J. Size, stability, and entrapment efficiency of phospholipid nanocapsules containing polypeptide antimicrobials. *J. Agric. Food Chem.,* **2003**, *51*(27), 8073-8079.

[56] Kosaraju, S.L., Tran, C., Lawrence, A. Liposomal delivery systems for encapsulation of ferrous sulfate: Preparation and characterization. *J. Liposome Res.,* **2006**, *16*(4), 347-358.

[57] Albaldawi, A.; Brennan, C.S.; Alobaidy, K.; Alammar, W.; Aljumaily, D. Effect of flour fortification with haem liposome on bread and bread doughs. *Int. J. Food Sci. Tech*., **2005**, *40*(8), 825-828.

[58] Walde, P.; Ichikawa, S. Enzymes inside lipid vesicles: Preparation, reactivity and applications. *Biomol. Eng*., **2001**, *18*(4), 143-177.

[59] Rodriguez-Nogales, J.M.; Pérez—Mateos, M.; Busto, M.D. Application of experimental design to the formulation of glucose oxidase encapsulation by liposomes. *J. Chem. Technol. Biotechnol.*, **2004**, *79*(7), 700-705.

[60] Kellaway, I.W.; Farr, S.J. Liposomes as drug delivery systems to the lung. *Adv. Drug Deliv. Rev.,* **1990**, *5*(1-2), 149-161.

[61] Smith, L.J.; Anderson, J.; Shamsuddin, M. Glutathione localization and distribution after intratracheal instillation: Implications for treatment. *Am. Rev. Respir. Dis*., **1992**, *145*(1), 153-159.

[62] Suntres, Z.E. Role of antioxidants in paraquat toxicity. *Toxicology,* **2002**, *180*(1), 65-77.

[63] Suntres, Z.E.; Shek, P.N. Incorporation of alpha-tocopherol in liposomes promotes the retention of liposome-encapsulated glutathione in the rat lung. *J. Pharm. Pharmacol*., **1994**, *46*, 23-28.

[64] Nacka, F.; Cansell, M.; Méléard, P.; Combe, N. Incorporation of α--tocopherol in marine lipid-based liposomes: *In vitro* and *in vivo* studies. *Lipids.,* **2001**, *36*(12), 1313-1320.

[65] Kirby, C.J. Controlled delivery of functional food ingredients: Opportunities for liposomes in the food industry. *Liposome Technol*., **1993**, *2*, 215-232.

[66] Mozafari, M.R.; Reed, C.J.; Rostron, C. Ultrastructural architecture of liposome-entrapped glutathione: A Cryo-SEM study. *Cell. Mol. Biol. Lett.,* **2004**, *9*(2 suppl.), 101-103.

[67] Acosta, E. Testing the effectiveness of nutrient delivery systems. In: *Delivery and controlled release of bioactives in foods and nutraceuticals*; Garti, N, Ed.; Woodhead Publishing Limited: England, **2008**; pp. 53-97.

[68] Fang, J.Y., Hwang, T.L., Huang, Y.L. Liposomes as vehicles for enhancing drug delivery via skin routes. *Curr. Nanosci*., **2006**, *2*(1), 55-70.

[69] Betz, G.; Aeppli, A.; Menshutina, N.; Leuenberger, H. *In vivo* comparison of various liposome formulations for cosmetic application. *Int. J. Pharm*., **2005**, *296*(1-2), 44-54.

[70] Moussaoui, N.; Cansell, M.; Denizot, A. MarinosomesÂ®, marine lipid-based liposomes: Physical characterization and potential application in cosmetics. *Inter. J. Pharm.,* **2002**, *242*(1-2), 361-365.

[71] Ziboh, V.A.; Miller, C.C.; Cho, Y. Metabolism of polyunsaturated fatty acids by skin epidermal enzymes: generation of antiinflammatory and antiproliferative metabolites. *Am. J. Clin. Nutr.,* **2000**, *71*, 361S-366S.

[72] Giacomoni, P.U. Advancement in skin aging: the future cosmeceuticals. *Clin. Dermatol.,* **2008**, *26*(4), 364-366.

[73] Chanchal, D.; Swarnlata S. Novel approaches in herbal cosmetics. *J. Cosmet. Dermatol.,* **2008**, *7*(2), 89-95.

[74] Calvagno, M.G.; Celia, C.; Paolino, D.; Cosco, D.; Iannone, M.; Castelli, F.; *et al*. Effects of lipid composition and preparation conditions on physical-chemical properties, technological parameters and *in vitro* biological activity of gemcitabine-loaded liposomes. *Curr. Drug Deliv.,* **2007**, *4*(1), 89-101.

[75] Spec, I.S.S.; Waterhouse, D.N.; Madden, T.D.; Cullis, P.R.; Bally, M.B.; Mayer, L.D.; Webb, M.S. In: *Liposomes, Methods in Enzymology*; Düzgüneş, N., Ed.; Elsevier Academic Press: USA, 2005; pp. 40-57.

[76] Uchegbu, I.F.; Florence, A.T. Non-ionic surfactant vesicles (niosomes)-physical and pharmaceutical chemistry. *Adv. Colloid. Interface. Sci.,* **1995**, *58*, 1-55.

[77] Uchegbu, I.F.; Vyas, S.P. Nonionic surfactant based vesicles (niosomes) in drug delivery. *Int. J. Pharm.,* **1998**, *172*, 33-70.

[78] Nagume, A.; Sato, Y.; Suzuki, Y. Electron spin resonance studies of phosphatidylcholine interacted with cholesterol and with a hopanoid in liposomal membrane. *Chem. Pharm. Bull.,* **1991**, *39*, 3071-3074.

[79] Coderch, L.; Fonollosa, J.; De Pera, M.; Estelrich, J.; De La Maza, A.; Parra J.L. Influence of cholesterol on liposome fluidity by EPR. Relationship with percutaneous absorption. *J. Control. Release,* **2000**, *68*(1), 85-95.

[80] Maruyama, K.; Ishida, O.; Kasaoka, S.; Takizawa, T.; Utoguchi, N.; Shinohara, A.; *et al*. Intracellular targeting of sodium mercaptoundecahydrododecaborate (BSH) to solid tumors by transferrin-PEG liposomes, for boron neutron-capture therapy (BNCT). *J. Control. Release,* **2004**, *98*(2), 195-207.

[81] Gensure, R.H.; Zeidel, M.L.; Hill, W.G. Lipid raft components cholesterol and sphingomyelin increase H +/OH- permeability of phosphatidylcholine membranes. *Biochem. J.,* **2006**, *398*(3), 485-495.

[82] Zucker, D.; Marcus, D.; Barenholz, Y.; Goldblum, A. Liposome drugs' loading efficiency: A working model based on loading conditions and drug's physicochemical properties. *J. Control. Release,* **2009**, *139*(1), 73-80.

[83] Huang, X.; Brazel, C.S. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. *J. Control. Release,* **2001**, *73*(2-3), 121-136.

[84] Meure, L.A.; Foster, N.R.; Dehghani, F. Conventional and dense gas techniques for the production of liposomes: A review. *AAPS Pharm. Sci. Tech.,* **2008**, *9*(3), 798-809.

[85] Lasch, J.; Weissig, V.; Brandl, D.M. In: *Liposomes: A practical approach*; Torchilin, V., Weissig, V., Eds.; Oxford University Press: USA, **2003**; pp. 3-29.

[86] Bangham, A.D.; Standish, M.M.; Watkins, J.C. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.,* **1965**, *13*(1), 238-252.

[87] Bangham, A.D., Standish, M.M., Weissmann, G. The action of steroids and streptolysin S on the permeability of phospholipid structures to cations. *J. Mol. Biol.,* **1965**, *13*(1), 253-259.

[88] Brunner, J.; Skrabal, P.; Hauser, H. Single bilayer vesicles prepared without sonication: physico chemical properties. *Biochim. Biophys. Acta – Biomembr.,* **1976**, *455*(2), 322-331.

[89] New, R.C.C. *Liposomes: A practical approach;* Oxford University Press: USA, **1990**; pp. 33-104.

[90] Batzri, S.; Korn, E.D. Single bilayer liposomes prepared without sonication. *Biochem. Biophys. Acta – Biomembr.,* **1973**, *298*(4), 1015-1019.

[91] Deamer, D.; Bangham, A.D. Large volume liposomes by an ether vaporization method. *Biochim. Biophys. Acta -Biomembr.,* **1976**, *443*(3), 629-634.

[92] Deamer, D.W. Preparation and properties of ether injection liposomes. *Ann. N. Y. Acad. Sci.,* **1978**, 308, 250-258.

[93] Jaafar-Maalej, C.; Diab, R.; Andrieu, V.; Elaissari, A.; Fessi, H. Ethanol injection method for hydrophilic and lipophilic drug-loaded liposome preparation. *J. Liposome Res.,* **2010**, *20*(3), 228-243.

[94] Hauschild, S.; Lipprandt, U.; Rumplecker, A.; Borchert, U.; Rank, A.; Schubert, R.; Förster, S. Direct preparation and loading of lipid and polymer vesicles using inkjets. *Small,* **2005**, *1*(12), 1177-1180.

[95] Szoka Jr, F.; Papahadjopoulos, D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci., USA,* **1978**, *75*(9), 4194-4198.

[96] Jahn, A.; Vreeland, W.N.; Devoe, D.L.; Locascio, L.E.; Gaitan, M. Microfluidic directed formation of liposomes of controlled size. *Langmuir,* **2007**, *23*(11), 6289-6293.

[97] Mortazavi, S.M.; Mohammadabadi, M.R.; Khosravi-Darani, K.; Mozafari, M.R. Preparation of liposomal gene therapy vectors by a scalable method without using volatile solvents or detergents. *J. Biotechnol.*, **2007**, *129*(4), 604-613.

[98] Mozafari, M.R.; Reed, C.J.; Rostron, C. Development of non-toxic liposomal formulations for gene and drug delivery to the lung. *Technol. Health Care.,* **2002**, *10*(3-4), 342-344.

[99] Mozafari, M.R. Liposomes: An overview of manufacturing techniques. *Cell. Mol. Biol. Lett.,* **2005**, *10*(4), 711-719.

[100] Anton, K.; Van Hoogevest, P.; Frederiksen, L. Preparation of a liposome dispersion containing an active agent by compression-decompression. EP616801. **1994**.

[101] Otake, K.; Imura, T.; Sakai, H.; Abe, M. Development of a new preparation method of liposomes using supercritical carbon dioxide. *Langmuir,* **2001**, *17*(13), 3898-3901.

[102] Castor ,T.P.; Chu, L. Methods and apparatus for making liposomes containing hydrophobic drugs. US5776486, **1998**.

[103] Bustami, R.T.; Chan, H-K.; Dehghani F.; Foster N.R. Recent applications of supercritical fluid technology to pharmaceutical powder systems. *Kona,* **2001**, *19*, 57-70.

[104] Frederiksen, L.; Anton, K.; Van Hoogevest, P.; Keller, H.R.; Leuenberger, H. Preparation of liposomes encapsulating water-soluble compounds using supercritical carbon dioxide. *J. Pharm. Sci.,* **1997**, *86*(8), 921-928.

[105] Frederiksen, L.; Anton, K.; Barratt, B.J.; Van Hoogevest, P.; Leuenberger, H. Use of supercritical carbon dioxide for preparation of pharmaceutical formulations. *3rd International Symposium on Supercritical Fluids*: Strasbourg, France, **1994**, *3*, pp. 235-240.

[106] Otake, K.; Shimomura, T.; Goto, T.; Imura, T.; Furuya, T.; Yoda, S.; Takebayashi, Y.; Sakai, H.; Abe, M. Preparation of liposomes using an improved supercritical reverse phase evaporation method. *Langmuir*, **2006**, *22*(6), 2543-2550.

[107] Barnadas-Rodríguez, R.; Sabés, M. Factors involved in the production of liposomes with a high-pressure homogenizer. *Int. J. Pharm.,* **2001**, *213*(1-2), 175-186.

[108] Bergstrand, N.; Arfvidsson, M.C.; Kim, J.M.; Thompson, D.H.; Edwards, K. Interactions between pH-sensitive liposomes and model membranes. *Biophys. Chem.,* **2003**, *104*(1), 361-379.

[109] Massing, U.; Cicko, S.; Ziroli, V. Dual asymmetric centrifugation (DAC)-A new technique for liposome preparation. *J. Control. Release,* **2008**, *125*(1), 16-24.

[110] Mayer, L.D.; Cullis, P.R.; Bally, M.B. The use of transmembrane pH gradient-driven drug encapsulation in the pharmacodynamic evaluation of liposomal doxorubicin. *J. Liposome Res.,* **1994**, *4*(1), 529-553.

[111] Gregoriadis, G. Liposome Technology, Liposome Preparation and Related Techniques. 3rd ed., Informa Healthcare Inc: USA, **2007**, Vol. III.

[112] Brandl, M.; Massing, U. In: *Liposome Technology Liposome Preparation and Related Technique;* Gregoriadis, G., Ed.; Informa Healthcare Inc: USA, 3rd ed, **2007**; Vol. I., pp. 241-260.

[113] Gregoriadis, G. Liposome Technology, Liposome Preparation and Related Techniques. 3rd ed., Informa Healthcare Inc: USA, **2007**, Vol. I.

[114] Li, C.; Deng, Y.J.; Cui, J.X. In: *Liposome Technology Liposome Preparation and Related Techniques*; Gregoriadis, G., Ed.*;* USA: Informa Healthcare Inc, 3rd ed, **2007**; Vol. I., pp. 35-53.

[115] Meerovich, G.; Meerovich I.; Lukyanets E.; Oborotova N.; Derkacheva V.; Smirnova, Z.; Loschenov, V.B.; Vorozhtsov, G.N.; Baryshnikov, A. Influence of liposome size on accumulation in tumor and therapeutic efficiency of liposomal near-IR photosensitizer for PDT based on aluminum hydroxide tetra-3-phenylthiophthalocyanine. Technical Proceedings of the NSTI Nanotechnology Conference and Trade Show, Quebec City: Canada, **2008**, pp. 41-44.

[116] Reimer, L. Scanning Electron Microscopy: Physics of Image Formation and Microanalysis; 2nd ed. Springer Series in Optical Sciences, **1998**.

[117] Spyratou, E.; Mourelatou, E.A.; Makropoulou, M.; Demetzos, C. Atomic force microscopy: A tool to study the structure, dynamics and stability of liposomal drug delivery systems. *Expert. Opin. Drug Deliv.,* **2009**, *6*(3), 305-317.

[118] Luykx, D.M.A.M.; Peters, R.J.B.; Van Ruth, S.M.; Bouwmeester, H. A review of analytical methods for the identification and characterization of nano delivery systems in food. *J. Agric. Food Chem.,* **2008**, *56*(18), 8231-8247.

[119] Edwards, K.; Baeumner, A.J. Analysis of liposomes. *Talanta,* **2006**, *68*(5), 1432-1441.

[120] Williams, T. Gel permeation chromatography: A review. *J. Mater. Sci.,* **1970**, *5*(9), 811-820.

[121] Grabielle-Madelmont, C.; Lesieur, S.; Ollivon, M. Characterization of loaded liposomes by size exclusion chromatography. *J. Biochem. Biophys. Meth.,* **2003**, *56*(1-3), 189-217.

[122] Taylor, K.M.G.; Morris, R.M. Thermal analysis of phase transition behaviour in liposomes. *Thermochim. Acta,* **1995**, *248*(C), 289-301.

[123] Sot, J.; Aranda, F.J.; Collado, M-I.; Goñi, F.M.; Alonso, A. Different effects of long- And short-chain ceramides on the gel-fluid and lamellar-hexagonal transitions of phospholipids: A calorimetric, NMR, and x-ray diffraction study. *Biophys. J.,* **2005**, *88*(5), 3368-3380.

[124] Saroglou, V.; Hatziantoniou, S.; Smyrniotakis, M.; Kyrikou, I.; Mavromoustakos, T.; Zompra, A.; Magafa, V.; Cordopatis, P.; Demetzos, C. Synthesis, liposomal formulation and thermal effects on phospholipid bilayers of leuprolide. *J. Pept. Sci.,* **2006**, *12*(1), 43-50.

[125] Nagahiro, I.; Mora, B.N.; Boasquevisque, C.H.R.; Scheule, R.K.; Patterson, G.A. Toxicity of cationic liposome-DNA complex in lung isografts. *Transplantation,* **2000**, *69*(9), 1802-1805.

[126] Filion, M.C.; Phillips, N.C. Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochim. Biophys. Acta - Biomembr.,* **1997**, *1329*(2), 345-356.

[127] Brgles, M.; Jurasin, D.; Sikiric, M.D.; Frkanec, R.; Tomasic, J. Entrapment of ovalbumin into liposomes - Factors affecting entrapment efficiency, liposome size, and zeta potential. *J. Liposome Res.,* **2008**, *18*(3), 235-248.

[128] Sulkowski, W.W.; Pentak, D.; Nowak, K.; Sulkowska, A. The influence of temperature, cholesterol content and pH on liposome stability. *J. Mol. Struct.,* **2005**, *744-747*(SPEC. ISS.), 737-747.

[129] Ogiso, T.; Niinaka, N.; Iwaki, M. Mechanism for enhancement effect of lipid disperse system on percutaneous absorption. *J. Pharm. Sci.,* **1996**, *85*(1), 57-64.

[130] Shimanouchi, T.; Ishii, H.; Yoshimoto, N.; Umakoshi, H.; Kuboi, R. Calcein permeation across phosphatidylcholine bilayer membrane: Effects of membrane fluidity, liposome size, and immobilization. *Colloids Surf. B Biointer.,* **2009**, *73*(1), 156-160.

[131] Johnson, S.M.; Bangham, A.D.; Hill, M.W.; Korn, E.D. Single bilayer liposomes. *Biochem. Biophys. Acta – Biomembr.,* **1971**, *223*(3), 820-826.

[132] Hope, M.J.; Bally, M.B.; Webb, G.; Cullis, P.R. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta – Biomembr.,* **1985**, *812*(1), 55-65.

[133] Mayer, L.D.; Hope, M.J.; Cullis, P.R. Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta - Biomembr.,* **1986**, *858*(1), 161-168.

[134] Yamauchi, M., Tsutsumi, K., Abe, M., Uosaki, Y., Nakakura, M., Aoki, N. Release of drugs from liposomes varies with particle size. *Biol. Pharm. Bull.,* **2007**, *30*(5), 963-936.

[135] Ruozi B.; Tosi G.; Leo E.; Vandelli M.A. Application of atomic force microscopy to characterize liposomes as drug and gene carriers. *Talanta,* **2007**, *73*(1), 12-22.

[136] Clerc, S.; Barenholz, Y. Liposome drug-loading method and composition, 5939096, **1997**.

[137] Laridi R.; Kheadr E.E.; Benech R.O.; Vuillermard J.C.; Lacroix C.; Fliss I. Liposome encapsulated nisin Z: optimization, stability and release during milk fermentation. *Inter. Dairy J.,* **2003**, *13*(4), 325-336.

[138] London, E.; Feigenson, G.W. A convenient and sensitive fluorescence assay for phospholipid vesicles using diphenylhexatriene. *Anal. Biochem.,* **1978**, *88*(1), 203-211.

[139] Zhang, X.M.; Patel A.B.; De Graaf, R.A.; Behar K.L. Determination of liposomal encapsulation efficiency using proton NMR spectroscopy. *Chem. Phys. Lipids,* **2004**, *127*(1), 113-120.

[140] Berger, N.; Sachse, A.; Bender, J.; Schubert, R.; Brandl, M. Filter extrusion of liposomes using different devices: Comparison of liposome size, encapsulation efficiency, and process characteristics. *Int. J. Pharm.,* **2001**, *223*(1-2), 55-68.

[141] Nii, T.; Takamura, A.; Mohri, K.; Ishii, F. Factors affecting physicochemical properties of liposomes prepared with hydrogenated purified egg yolk lecithins by the microencapsulation vesicle method. *Colloids. Surf. B: Biointerf.,* **2003**, *27*(4), 323-332.

[142] Montero, M.T.; Saiz, D.; Sitges, R.; Vàzquez, J.L.; Borrell, J.H. Influence of physicochemical properties of fluoroquinolones on encapsulation efficiency in liposomes. *Int. J. Pharm.,* **1996**, *138*(1), 113-120.

[143] Schneider, T.; Sachse, A.; Robling, G.; Brandl, M. Generation of contrast-carrying liposomes of defined size with a new continuous high pressure extrusion method. *Int. J. Pharm.,* **1995**, *117*(1), 1-12.

[144] Oku, N.; Kendall, D.A.; Macdonald, R.C. A simple procedure for the determination of the trapped volume of liposomes. *Biochim. Biophys. Acta,* **1982**, *691*(2), 332-340.

[145] Anzai, K.; Yoshida, M.; Kirino,Y. Change in intravesicular volume of liposomes by freeze-thaw treatment as studied by the ESR stopped-flow technique. *Biochim. Biophys. Acta - Biomembr.,* **1990**, *1021*(1), 21-26.

[146] Hinton, D.P.; Johnson, Jr C.S. Simultaneous measurement of vesicle diffusion coefficients and trapping efficiencies by means of diffusion ordered 2D NMR spectroscopy. *Chem. Phys. Lipids,* **1994**, *69*(2), 175-178.

[147] Acosta, E. In: *Delivery and controlled release of bioactives in foods and nutraceuticals*; Garti, N., Ed.; Woodhead Publishing Limited: USA, **2008**; pp. 53-97.

[148]   Hauser, H. In: *Liposome Technology, Liposome Preparation and Related Techniques;* Gregoriadis, G. Ed.; CRC Press: USA; **1993**; pp. 197-251.

[149]   Crowe, J.H.; Crowe, L.M.; Oliver, A.E.; Tsvetkova, N.M.; Wolkers, W.F.; Tablin, F. The trehalose myth revisited: introduction to a symposium on stabilization of cells in the dry state. *Cryobiology*, **2001**, *43*, 89-105.

[150]   Crowe, L.M.; Womersley, C.; Crowe, J.H.; Reid, D.; Appel, L.; Rudolph A. Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates. *Biochim. Biophys. Acta - Biomembr.,* **1986**, *861*(C), 131-140.

[151]   Payne, N.I.; Timmins, P.; Ambrose, C.V. Proliposomes: A novel solution to an old problem. *J. Pharm. Sci.,* **1986**, *75*(4), 325-329.

[152]   Payne, N.I.; Browning, I.; Hynes, C.A. Characterization of proliposomes. *J. Pharm. Sci.,* **1986**, *75*(4), 330-333.

[153]   Perrett, S.; Golding, M.; Williams, W.P. A simple method for the preparation of liposomes for pharmaceutical applications: Characterization of the liposomes. *J. Pharm. Pharmacol.,* **1991**, *43*(3), 154-161.

[154]   Smith, L.L. Another cholesterol hypothesis: Cholesterol as antioxidant. *Free Radic. Biol. Med.*, **1991**, *11*, 47-61.

[155]   Zuidam, N.J.; Lee, S.S.L.; Crommelin, D.J.A. Sterilization of liposomes by heat treatment. *Pharm. Res.,* **1993**, *10*(11), 1591-1596.

[156]   Zuidam, J.N.; De Vruech, R.; Crommelin, D.J.A. Characterization of liposomes. In: *Liposomes A Practical Approach*; Torchilin, V.P., Weissig, V., Eds; 2nd ed, Oxford University Press: USA, **2003**; pp. 50-53.

[157]   Sas, B.; Peys, E.; Helsen, M. Efficient method for (lyso)phospholipid class separation by high-performance liquid chromatography using an evaporative light-scattering detector. *J. Chromatogr.,* **1999**, *864*, 179-182.

[158]   Vernooij, E.A.A.M.; Kettenes-Van Den Bosch J.J.; Crommelin, D.J.A. Rapid determination of acyl chain position in egg phosphatidylcholine by high performance liquid chromatography/electrospray mass spectrometry. *Rapid Commun. Mass Spectrom.,* **1998**, *12*(2), 83-86.

[159]   Tchoreloff, P.; Gulik, A.; Denizot, B.; Proust, J.E.; Puisieux, F. A structural study of interfacial phospholipid and lung surfactant layers by transmission electron microscopy after Blodgett sampling: Influence of surface pressure and temperature. *Chem. Phys. Lipids,* **1991**, *59*(2), 151-165.

[160]   Düzgüneş, N. *Liposomes, Methods in Enzymolog;* Elsevier Academic Press: USA, **2004**.

[161]   Lasic, D.D. *Liposomes, From Physics to Applications;* Elsevier Science Publishers: Amsterdam, **1993**.

[162]   Barenholz, Y.; Crommelin, D.J.A. In: *Boylan Encyclopedia of Pharmaceutical Technology;* Swarbrick, J.C., Ed.; Dekker: USA, **1994**; pp.1-39.

[163]   Samuni, A.M.; Lipman, A.; Barenholz, Y. Damage to liposomal lipids: Protection by antioxidants and cholesterol- mediated dehydration. *Chem. Phys. Lipids,* **2000**, *105*, 121-134.

[164]   Halliwell, B.; Gutteridge, J.M.C. *Free Radicals in Biology and Medicine;* Clarendon Press: USA, **1989**.

[165]   Lang, J.K.; Vigo-Pelfrey C. Quality control of liposomal lipids with special emphasis on peroxidation of phospholipids and cholesterol. *Chem. Phys. Lipids.,* **1993**, *64*, 19-29.

[166]   Higashiyama, T. Novel functions and applications of trehalose. *Pure Appl. Chem.,* **2002**, *74*(7), 1263-1269.

[167]   Benaroudj, N.; Lee, D.H.; Goldberg, A.L. Trehalose Accumulation during Cellular Stress Protects Cells and Cellular Proteins from Damage by Oxygen Radicals. *J. Biol. Chem.,* **2001**, *276*(26), 24261-24267.

[168]   Chen, Q.; Haddad, G.G. Role of trehalose phosphate synthase and trehalose during hypoxia: From flies to mammals. *J. Exp. Biol.,* **2004**, *207*(18), 3125-3129.

[169]   Chen, Q.; Enbo, M.A.; Behar, K.L.; Xu, T.; Haddad, G.G. Role of trehalose phosphate synthase in anoxia tolerance and development in Drosophila melanogaster. *J. Biol. Chem.,* **2002**, *277*(5), 3274-3279.

[170]   Neta, T.; Takada, K.; Hirasawa, M. Low-cariogenicity of trehalose as a substrate. *J. Dent.,* **2000**, *28*(8), 571-576.

[171]   Komes, D.; Lovrić, T.; Ganić, K.K.; Gracin, L. Study of trehalose addition on aroma retention in dehydrated strawberry puree. *Food Technol. Biotechnol.,* **2003**, *41*(2), 111-119.

[172]   Grit, M.; Zuidam, N.J.; Crommelin, D.J.A. Analysis and hydrolysis kinetics of phospholipids in aqueous liposome dispersions. *Liposome Technol.,* **1993**, 1, 455-486.

[173]   Grit, M.; Crommelin, D.J.A. Chemical stability of liposomes: Implications for their physical stability. *Chem. Phys. Lipids.,* **1993**, *64*(1-3), 3-18.

[174]   Frojaer, S.; Hjorth, E.L.; Worts, O. Stability and storage of liposomes. Optimization of Drug Delivery, Alfred Benzon Symposium: Copenhagen, Denmark, **1982**; pp. 384-404.

[175]   Grit, M.; Underberg, W.J.M.; Crommelin, D.J.A. Hydrolysis of saturated soybean phosphatidylcholine in aqueous liposome dispersions. *J. Pharm. Sci.,* **1993**, *82*(4), 362-366.

[176]   Shmeeda, H.; Even-Chen, S.; Honen, R.; Cohen, R.; Weintraub, C.; Barenholz, Y. In: *Liposomes, Methods in Enzymology*; Düzgüneş, N., Ed.; Elsevier Academic Press, USA, **2003**; pp.279-92.

[177]   Mozafari, M.R. *Nanocarrier Technologies: Frontiers of Nanotherapy*; The Netherlands: Springer. **2006**.

[178]   Stephenson, S.M.; Low, P.S.; Lee, R.J. In: *Liposomes, Methods in Enzymology;* Düzgüneş, N., Ed.; Elsevier Academic Press: USA, **2004**; Vol. *387*, pp. 33- 50.

[179]   Sunamoto, J.; Sato, T.; Hirota, M.; Fukushima, K.; Hiratani, K.; Hara, K. A newly developed immunoliposome - an egg phosphatidylcholine liposome coated with pullulan bearing both a cholesterol moiety and an IgMs fragment. *Biochim. Biophys. Acta -Biomembr.,* **1987**, *898*, 323-3230.

[180]   Cirli, O.O.; Hasirci, V. UV-induced drug release from photoactive REV sensitized by suprofen. *J. Control. Release,* **2004**, *96*(1), 85-96.

[181]   Babincová, M.; Sourivong, P.; Chorvát, D.; Babinec, P. Laser triggered drug release from magnetoliposomes. *J. Magn. Magn. Mater.,* **1998**, *194*(1), 163-166.

[182]   Pitt, W.G.; Husseini, G.A. Ultrasound in drug and gene delivery. *Adv. Drug Deliv. Rev.,* **2008**, *60*(10), 1095-1096.

[183]   Enden, G.; Schroeder, A. A mathematical model of drug release from liposomes by low frequency ultrasound. *Ann. Biomed. Eng.,* **2009**, 1-6.

[184]   Fortin-Ripoche, J-P.; Martina, M.S.; Gazeau, F.; Ménager, C.; Wilhelm, C.; Bacri, J-C.; Lesieur, S.; Clément, O. Magnetic targeting of magnetoliposomes to solid tumors with MR imaging monitoring in mice: Feasibility. *Radiology*, **2006**, *239*(2), 415-424.

[185]   Zheng, X.; Lu, J.; Deng, L.; Xiong, Y.; Chen, J. Preparation and characterization of magnetic cationic liposome in gene delivery. *Int. J. Pharm.,* **2009**, *366*(1-2), 211-217.

[186]   Liang, X.; Wang, H.; Jiang, X.; Chang, J. Development of monodispersed and functional magnetic polymeric liposomes via simple liposome method. *J. Nanopart. Res.,* **2010**, *12*(5), 1723-1732.

[187]   Asmatulu, R.; Fakhari, A.; Wamocha, H.L.; Hamdeh, H.H.; Ho, JC. Fabrication of magnetic nanocomposite spheres for targeted drug delivery. ASME International Mechanical Engineering Congress and Exposition: Proceeding, Boston, **2009**; pp. 195-198.

[188]   Kanwar, J.R.; Mahidhara, G.; Kanwar, R.K. Recent advances in nanoneurology for drug delivery to the brain. *Curr. Nanosci.,* **2009**, *5*(4), 441-448.

[189]   Zhou, L.; Yuan, J.; Yuan, W.; Sui, X.; Wu, S.; Li, Z.; Shen, D. Synthesis, characterization, and controllable drug release of pH-sensitive hybrid magnetic nanoparticles. *J. Magn. Magn. Mater.,* **2009**, *321*(18), 2799-2804.

[190]   Wan, Y.; Angleson, J.K.; Kutateladze, A.G. Liposomes from novel photolabile phospholipids: Light-induced unloading of small molecules as monitored by PFG NMR. *J. Am. Chem. Soc.,* **2002**, *124*(20), 5610-5611.

[191]   Miller, C.R.; Clapp, P.J.; O'Brien, D.F. Visible light-induced destabilization of endocytosed liposomes. *FEBS Lett.* **2000**, *467*(1), 52-56.

[192]   Lei, Y.; Hurst, J.K. Photoregulated potassium ion permeation through dihexadecyl phosphate bilayers containing azobenzene and stilbene surfactants. *Langmuir*, **1999**, *15*(10), 3424-3429.

[193]   Huth, U.S.; Schubert, R.; Peschka-Suss, R. Investigating the uptake and intracellular fate of pH-sensitive liposomes by flow cytometry and spectral bio-imaging. *J. Control. Release,* **2006**, *110*(3), 490-504.

[194]   Hee, D.H.; Tae, W.K.; Byung, C.S.; Ho, S.C. Release of calcein from temperature-sensitive liposomes in a poly(N-isopropylacrylamide) hydrogel. *Macromol. Res.,* **2005**, *13*(1), 54-61.

[195]   Kono, K. Thermosensitive polymer-modified liposomes. *Adv. Drug Deliv. Rev.,* **2001**, *53*(3), 307-319.

[196]   Kono K.; Takagishi T. Temperature-sensitive liposomes. Methods Enzymol. **2004**, *387*, 73-82.

[197]   Li, J.; Wang, B.; Liu, P. Possibility of active targeting to tumor by local hyperthermia with temperature-sensitive nanoparticles. *Med. Hypotheses,* **2008**, *71*(2), 249-521.

[198]   Nobs, L.; Buchegger, F.; Gurny, R.; Allémann, E. Current methods for attaching targeting ligands to liposomes and nanoparticles. *J. Pharm. Sci.,* **2004**, *93*(8), 1980-1992.

[199]   Vasir, J.K.; Reddy, M.K.; Labhasetwar, V.D. Nanosystems in drug targeting: opportunities and challenges. *Curr. Nanosci.,* **2005**, *1*(1), 47-64.

[200]   Petrak, K. Essential properties of drug-targeting delivery systems. *Drug Discov. Today,* **2005**, *10*(23-24), 1667-173.

[201]   Byrne, J.D.; Betancourt, T.; Brannon-Peppas, L. Active targeting schemes for nanoparticle systems in cancer therapeutics. *Adv. Drug Deliv. Rev.,* **2008**, *60*(15), 1615-1626.

[202]   Almeida, J.D.; Brand, C.M.; Edwards, D.C.; Heath, T.D. Formation of virosomes from influenza subunits and liposomes. *Lancet,* **1975**, *2*(7941), 899-901.

[203]   KaRa, J.; Mach, O.; Cerna, H. Replication of rous sarcoma virus and the biosynthesis of the oncogenic subviral ribonucleoprotein particles ("viro-

somes") in the mitochondria isolated from rous sarcoma tissue. *Biochem. Biophys. Res. Commun.,* **1971**, *44*(1), 162-170.

[204] Adamina, M.; Guller, U.; Bracci, L.; Heberer, M.; Spagnoli, G.C.; Schumacher, R. Clinical applications of virosomes in cancer immunotherapy. *Expert Opin. Biol. Ther.,* **2006**, *6*(11), 1113-1121.

[205] Huang, A.; Kennel, S.J.; Huang, L. Immunoliposome labeling: A sensitive and specific method for cell surface labeling. *J. Immunol. Methods.,* **1981**, *46*(2), 141-151.

[206] Mizoue, T.; Horibe, T.; Maruyama, K.; Takizawa, T.; Iwatsuru, M.; Kono, K.; Yanagie, H.; Moriyasu, F. Targetability and intracellular delivery of anti-BCG antibody-modified, pH-sensitive fusogenic immunoliposomes to tumor cells. *Int. J. Pharm.,* **2002**, *237*(1-2), 129-137.

[207] Stolnik, S.; Illum, L.; Davis, S.S. Long circulating microparticulate drug carriers. *Adv. Drug Deliv. Rev.,* **1995**, *16*(2-3), 195-214.

[208] Hashizume, H.; Baluk, P.; Morikawa, S.; McLean, J.W.; Thurston, G.; Roberge, S.; Jain, R.K.; McDonald, D.M. Openings between defective endothelial cells explain tumor vessel leakiness. *Am. J. Pathol.,* **2000**, *156*(4), 1363-1380.

[209] Takeuchi, H.; Kojima, H.; Yamamoto, H.; Kawashima, Y. Passive targeting of doxorubicin with polymer coated liposomes in tumor bearing rats. *Biol. Pharm. Bull.,* **2001**, *24*(7), 795-799.

[210] Allen, T.M. Long-circulating (sterically stabilized) liposomes for targeted drug delivery. *Trends Pharmacol. Sci.,* **1994**, *15*(7), 215-220.

[211] Lim, S.K.; Park, H.J.; Choi, E.K.; Kim, J.S. Long-circulating, temperature-sensitive and EGFR-targeted liposomes for drugs delivery. *Key Eng. Mater.,* **2007**, *342-343*, 537-540.

[212] Wang, J.S.; Zhu, J.B. Study on Azithromycin liposomes obtained by the ether injection method. *Chin. Pharm. J.,* **2005**, *40*(11), 874-876.

[213] Rojanapanthu, P.; Sarisuta, N.; Chaturon, K.; Kraisintu, K. Physicochemical properties of amphotericin B liposomes prepared by reverse-phase evaporation method. *Drug Dev. Ind. Pharm.,* **2003**, *29*(1), 31-37.

[214] Winterhalter, M.; Lasic, D.D. Liposome stability and formation: Experimental parameters and theories on the size distribution. *Chem. Phys. Lipids.,* **1993**, *64*(1-3), 35-43.

[215] Sakai, H.; Gotoh, T.; Imura, T.; Sakai, K.; Otake, K.; Abe, M. Preparation and properties of liposomes composed of various phospholipids with different hydrophobic chains using a supercritical reverse phase evaporation method. *J. Oleo. Sci.,* **2008**, *57*(11), 613-621.

[216] Foster, N.; Mammucari, R.; Dehghani, F.; Barrett, A.; Bezanehtak, K.; Coen, E.; *etal*. Processing Pharmaceutical Compounds Using Dense Gas Technology. *Ind. Eng. Chem. Res.,* **2003**, *42*(25), 6476-6493.

[217] Barnadas i Rodríguez, R.; Sabés i Xamaní, M. In: *Liposome ,Methods in Enzymology;* Düzgüneş, N.; Elsevier Academic Press: USA, **2003**; Vol. *367*, pp. 28-46.

[218] Li, Z.; Qin, B.; Zhang, Z.Q.; Zhang, L.Y.; Yao, J. Effect of liposome encapsulation on lrinotecan lactone ring and on drug release *in vitro*. *Chin. Pharm. J.,* **2008**, *43*(13), 1003-1007.