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Liposomes as Potential Drug Carrier Systems for Drug Delivery

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1. Introduction

Lipids are amphiphilic molecules, where one part of the molecule is water-loving (hydrophilic) and the other water-hating (hydrophobic). When lipids are placed in contact with water, the unfavorable interactions of the hydrophobic segments of the molecule with the solvent result in the self assembly of lipids, often in the form of liposomes. Liposomes consist of an aqueous core surrounded by a lipid bilayer, much like a membrane, separating the inner aqueous core from the bulk outside. They were first discovered by Bangham and his co-workers in 1961 [1] and described as swollen phospholipid systems [2]. In the following years, a variety of enclosed phospholipid bilayer structures were defined which were initially called bangosomes and then liposomes, which was derived by the combination of two Greek words, “lipos” meaning fat and “soma” meaning body.

Liposomes have been used to improve the therapeutic index of new or established drugs by modifying drug absorption, reducing metabolism, prolonging biological half-life or reducing toxicity. Drug distribution is then controlled primarily by properties of the carrier and no longer by physico-chemical characteristics of the drug substance only.

Lipids forming liposomes may be natural or synthetic, and liposome constituents are not exclusive of lipids, new generation liposomes can also be formed from polymers (sometimes referred to as polymersomes). Whether composed of natural or synthetic lipids or polymers, liposomes are biocompatible and biodegradable which make them suitable for biomedical research. The unique feature of liposomes is their ability to compartmentalize and solubilize both hydrophilic and hydrophobic materials by nature. This unique feature, coupled with biocompatibility and biodegradability make liposomes very attractive as drug delivery vehicles.

Hydrophobic drugs place themselves inside the bilayer of the liposome and hydrophilic drugs are entrapped within the aqueous core or at the bilayer interface. Liposomal formulations enhance the therapeutic efficiency of drugs in preclinical models and in humans compared to conventional formulations due to the alteration of biodistribution. Liposome binding drugs, into or onto their membranes, are expected to be transported without rapid degradation and minimum side effects to the recipient because generally liposomes are composed of biodegradable, biologically inert and non-immunogenic lipids. Moreover, they produce no pyrogenic or antigenic reactions and possess limited toxicity [3-5]. Consequently, all these properties as well as the ease of surface modification to bear the targetable properties make liposomes more attractive candidates for use as drug-delivery vehicles than other drug carrying systems such as nanoparticles [6, 7] and microemulsions [8, 9]. In the 1970s [1, 10-13], liposomes were introduced as drug delivery vehicles but the initial clinical results were not satisfactory due to their colloidal and biological instability and their inefficient encapsulation of drug molecules.

Subsequent research on their stability and drug interactions resulted in several commercial liposome products in the market in the 1980s and early 1990s [14]. A schematic representation of liposomal drug delivery is given in Figure 1.

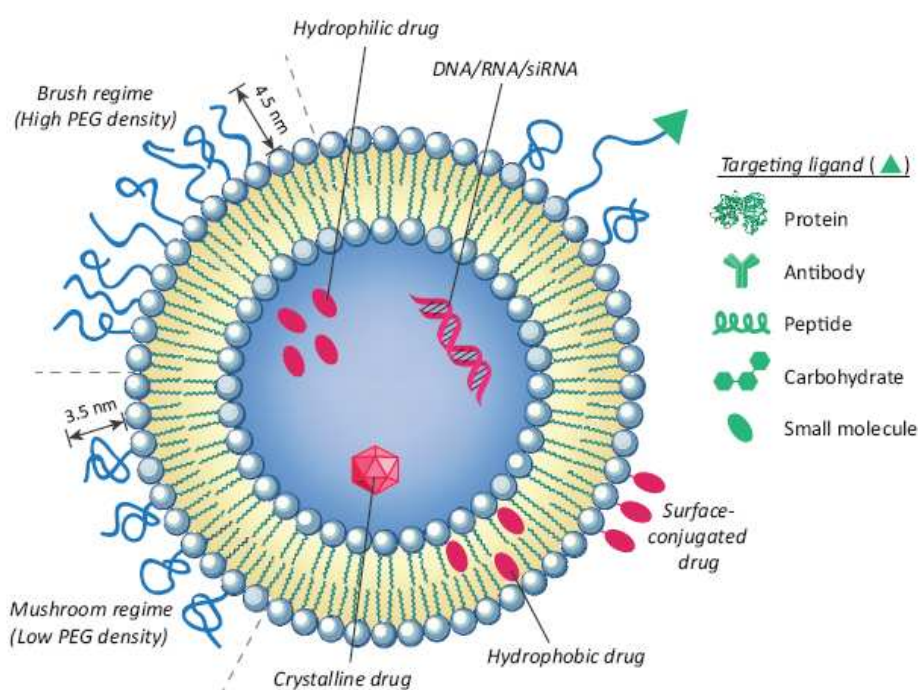


Figure 1. Structural and design considerations for liposomal drug delivery. Liposomes can be surface functionalized to endow stealth through PEGylation and to promote receptor-mediated endocytosis by using targeting ligands such as antibodies, peptides, proteins, carbohydrates, and various other small molecules. PEGylation extends liposomal circulation half-life in vivo by reducing clearance, immune recognition, and the non-specific absorption of serum proteins. Polyethylene glycol (PEG) density determines its structure at the liposome surface, with densities below 9% adopting a mushroom-like globular structure and those above 9% adopting a more rigid, extended, brush-like morphology. Chemotherapeutics or diagnostics can be encapsulated into the aqueous lumen, incorporated into the lipid bilayer, or conjugated to the liposome surface. Abbreviation: siRNA, small interfering RNA [15].

Liposomes represent versatile and advanced nanodelivery systems for a wide range of biologically active compounds [16]. The final amount of the encapsulated drug is affected by a selection of an appropriate preparation method providing a preparation of liposomes of various size, lamellarity and physicochemical properties [17]. The entrapment of the drugs, both hydrophilic and hydrophobic, into the liposomes is used to bypass the frequent generic toxicity associated with the drug as often seen in cancer drugs [18]. Thus, it represents a very effective route that enhances the drug therapeutic effect. The modification of liposomes permits a passive or active targeting of the tumor site. This effect enables an efficient drug payload into the malignant cell of tumors, while the non-malignant cells become minimally impacted.

In some of the first demonstrations of the improved *in vivo* activity of liposome-encapsulated drugs in animal models, the anti-cancer drug cytosine arabinoside used which showed a significant increase in the survival times of the mice bearing leukemia [19, 20] which became a popular model for testing the effects of a wide range of liposome characteristics on therapeutic outcomes. Following experiments include liposomal amphotericin B [21] and liposomal doxorubicin [22] that finally led to the first clinical trials of liposomal drugs. Nowadays; the liposomal products (as a suspension, as an aerosol or in a semi-solid form such as a gel, cream, or powder) in the market still include mostly anticancer preparations as well as antifungal and antibacterial preparations and cosmetics. In addition, liposomes are recently used as therapeutic agents to treat a disease because increased gene transfer efficiencies have been obtained via liposomal gene vectors in gene therapy.

The benefits and limitations of liposome drug carriers critically depend and based on physicochemical and colloidal characteristics such as size, composition, loading efficiency and stability, as well as their biological interaction with the cell membranes. There are four major interactions between liposomes and cells [23]. The predominant interaction among them is either simple adsorption or subsequent endocytosis. Adsorption occurs when the attractive forces exceed the repulsive ones and obviously this type of interaction depends on the surface properties of liposomes. In the delivery through endocytosis, liposome and its contents indirectly place themselves in the cytoplasm. Fusion with cell membranes, delivery of the liposomal content directly into the cell through the merge of liposome lipids into the membrane, is much rarer. The last possible interaction is the lipid exchange which is a long-range interaction that involves the exchange of bilayer constituents, such as lipids, cholesterol, and membrane bound molecules with components of cell membranes. Upon entering into the body, the delivered liposomes via one of these interaction types trigger the response of the immune system and the encapsulated material may become inactive. Therefore; substantial researches have been carried out in the development of the biocompatible and nonrecognizable liposomal surfaces.

Various types of liposomes can be prepared by different preparation methods, depending on the required application. In this chapter, these methods are summarized to give a general understanding of the relationship between structure and functionality of liposomes. As one of the advantages of liposomal formulations is the encapsulation ability of both hydrophobic and hydrophilic drugs, incorporation methods are shortly visited. Physical properties of liposomes

such as stability, storage and sterilization are discussed along with the characterization techniques for size, charge, etc. Clinical applications of liposomes are a vast area of research where cancer therapy is the area of highest impact. Different clinical applications of liposomes and most recent advances in cancer therapy are summarized. New generation involving constituents other than conventional ones such as phospholipids prove to be a growing field in nanotechnology. A brief list of different types of new generation liposomes are given with short descriptions at the end of this chapter.

2. Liposome preparation methods

The manufactured liposome features are directly related to the preparation method. Although liposome formation may be spontaneous, often some mechanical agitation is required. In order to have control over the size and structure of the liposomes that are formed, increase the efficiency of entrapment of the desired molecules, and prevent subsequent leakage from the liposomes, different preparation methods have been devised.

There are a few parameters that should be considered during the method selection: 1) the physicochemical characteristics of the material to be entrapped and those of the liposomal ingredients, 2) the nature of the medium in which the liposomes are dispersed, 3) the effective concentration of the encapsulated material and its potential toxicity, 4) additional processes involved during application (delivery of the liposomes), 5) optimum size, polydispersity and shelf-life of the liposomes for the intended application and 6) batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products [24-26].

Liposome size is a crucial parameter in determining the circulation half-life of liposomes in drug delivery. The amount of encapsulated drug is also related with the size and the number of bilayers of the prepared liposome. According to the desired formulation, different liposome preparation methods can be employed. The main difference in these methods is their approach to overcome the low solubility of lipids in water. Accordingly, these methods can be classified as mechanical agitation, solvent evaporation, solvent injection, and detergent solubilization. In all the above mentioned methods, drug loading is passive.

2.1. Mechanical agitation

In this method, lipids are directly solubilized in water upon application of high mechanical agitation, through the use of probe sonication. It is one of the simplest methods of liposome preparation, however, yields small liposomes that are highly unstable in terms of their size and suffers from the drawback that it is impossible to remove completely the risk of lipid degradation by contact with the hot probe, and contamination with titanium from the probe. Its advantage is the exclusion of use of organic solvents as described in the following methods. However, for drug delivery applications, liposomes prepared with mechanical agitation are not suitable due to their size instability and high leakage of encapsulated drugs [27].

2.2. Solvent evaporation

In general this method consists of four major steps; first is the solubilization of the lipid (and a hydrophobic compound) in an organic solvent; second is solvent evaporation; third is hydration with a buffer (and the hydrophilic compound) and if need the fourth often involves obtaining unilamellar liposomes from the obtained multilamellar ones.

The aqueous volume enclosed within these lipid membranes is very small proportion of total volume used for preparation (5-10%). Consequently, large amount of water soluble drug is wasted during the preparation. On the other hand, lipid soluble drug can be encapsulated with 100% efficiency, providing that they are not present in quantities which overwhelm the structural components of the membrane [28]. The volume of entrapment can be significantly increased by the usage of negatively charged lipids in the membrane which tend to push the bilayers apart from each other. The same effect can also be achieved in the presence of neutral lipids by freezing and thawing repeatedly the obtained liposomes. 30% volume of entrapment can be achieved, which can further be increased at higher lipid concentrations [29]. The freeze-thaw protocol results in a dramatic change in liposome morphology followed by freeze-fracture electron micrographs. Before freeze-thawing, the samples exhibit the tightly packed “onions skin” arrangements of concentric bilayers normally associated with liposomal systems. After a few freeze-thaw steps, however, new structures are observed where the interlamellar spaces are much increased, and where closed lamellar systems can be intercalated between bilayers [30].

2.2.1. Solubilization of the lipid

The starting point of this liposome preparation method is to prepare an organic solution of membrane lipids in order to ensure complete and homogenous mixing of all the components as they are required in the final membrane preparation. Compounds to be incorporated which are lipid soluble will be added to the organic solution, while compounds to be entrapped in the aqueous compartment of liposomes will be dissolved in the aqueous environment. In this method, phospholipids are first dissolved in an organic solvent along with lipid soluble compounds (if any) to be incorporated in the liposome to ensure complete and homogenous mixing.

2.2.2. Solvent evaporation

The next step is the evaporation of the organic solvent. The simplest is to allow the solvent to evaporate in a glass container. A better method is evaporating the solvent using a rotary evaporator connected to a vacuum pump to obtain a thin film of the lipid on the walls of a round bottom flask. In order to increase encapsulation, it is recommended to start with a large volume round bottom flask so that the lipids will be dried down onto a large surface area possible to form a very thin film. The evaporator is detached from vacuum pump and introduced to nitrogen. The container is then removed from the evaporator and fixed to a lyophilizer or exposed to high vacuum overnight to remove the residual solvent.

An alternative method of dispersing the lipids in a finely-divided form before the addition of aqueous media is to freeze-dry the dissolved lipids in an organic solvent [31]. The important concept in this method is the choice of the organic solvent which should have a freezing point above the temperature of the condenser of the freeze-drying and also be inert with regard to rubber seals of commercial lyophilizers. When these restrictions are concerned, the most suitable organic solvent happens to be tertiary butanol.

2.2.3. Hydration

Evaporation (or freeze-drying) of the solvent is followed by hydration of lipids with the aqueous medium. Often for hydration, a suitable buffer at a temperature above the phase transition temperature of the phospholipid is employed. The solution is swirled manually or mechanically (either with a bath sonicator or vortex mixer) until all the lipids have been incorporated into the solution. The resulting product is a milky suspension of lipids which is allowed to stand for a while for the complete swelling to give MLVs [28]. Further treatment is required for the preparation of ULVs, which will be discussed later in the text.

It is possible to obtain LUVs instead of MLVs during hydration by introducing an aqueous sucrose solution down the side of the flask by inclining the flask to one side and slowly returning the flask to the upright orientation, allowing the fluid to run gently over the lipid layer on the bottom of the flask. The swelling is carried out as usual without any shaking or agitation. The suspension is then centrifuged and the layer of MLVs floating on the surface is removed, leaving LUVs in solution.

2.2.4. Obtaining SUVs from MLVs

After preparation of MLVs by hydration of dried lipid, it is possible to continue processing the liposomes in order to modify their size and other characteristics. For many purposes, MLVs are too large of too heterogeneous population to work with. There are several methods devised to reduce their size. These include techniques such as micro-emulsification, extrusion, and ultrasonication. A second set of methods is designed to increase the entrapment volume of hydrated lipids, and/or reduce the lamellarity of the liposomes formed, and involves procedures such as freeze-drying, freeze-thawing or induction of vesiculation by ions or pH change.

Microemulsification of liposomes is performed with an equipment called micro fluidizer to prepare small vesicles from concentrated lipid suspension. This method can produce liposomes in 50-200 nm size range with the encapsulation efficiency of up to 75% [32].

Sonication [33] disrupts MLV suspensions by using sonic energy to produce SUVs with diameters in the range of 15-50 nm. There are two methods of sonication; bath sonication and probe sonication. The former method is used for large volumes of dilute lipids whereas the latter one is used for suspensions which require high energy, such as high concentration of lipid suspensions. The disadvantage of probe sonication is the contamination of preparation with metal from the tip of the probe which should be removed by centrifugation prior to use. Also, as a result of high energy, probe sonication suffers from overheating the lipid suspension

causing degradation. For these reasons, bath sonicators are the most widely used instrumentation for SUV preparation.

An even gentler method of reducing the size of the liposomes is to pass through a membrane filter of defined pore size [34]. This can be at much more lower pressure and can give populations in which one can choose the upper size limit depending on the exact pore size of the filter used. This membrane extrusion technique can be used to process both LUVs and MLVs in which liposome contents are exchanged with the suspending medium during breaking and resealing of the phospholipid bilayers as they pass through the polycarbonate membrane. In order to achieve as high an entrapment as possible of water-soluble compounds, it is crucial to have these compounds present in the suspending medium during the extrusion. An almost completely unilamellar population can be produced after 5-10 repeated extrusions through two stacked membranes.

In freezing-thawing method, SUVs are rapidly frozen and thawed slowly. The short-lived sonication disperses aggregated materials to LUV. The creation of unilamellar vesicles is as a result of the fusion of SUV throughout the processes of freezing and thawing [35-37].

2.3. Solvent injection

In this type of preparation methods, lipids are first dissolved in an organic solvent and then brought into contact with the aqueous phase containing the materials to be encapsulated within the liposome. The lipids align themselves into a monolayer at the interface between the organic and aqueous phase which is an important step to form the bilayer of the liposome [29]. There are three categories in solvent dispersion method including; (i) a miscible organic solvent with the aqueous phase, (ii) an immiscible organic solvent with the aqueous phase that is used in excess, and (iii) an immiscible organic solvent used in excess with the aqueous phase.

2.3.1. Ethanol injection method

In this method an ethanol solution of lipids is injected rapidly into an excess saline or other aqueous medium by a fine needle [38]. The injection force is usually sufficient to achieve complete mixing, so that ethanol is diluted in water, and lipids are dispersed evenly throughout the medium. This method yields a high proportion of SUVs. This method is extremely simple and it has a very low risk of degradation for sensitive lipids. Its major disadvantages are the limitation of solubility of lipids in ethanol and the volume of ethanol that can be introduced into the medium, which in turn limits the quantity of lipid dispersed, so that the resulting liposome solution is generally dilute. As a result, the percentage encapsulation for hydrophilic materials is very low. One last disadvantage for this method is the difficulty of the removal of ethanol from the lipid membranes.

2.3.2. Ether injection method

This method [39, 40] involves injecting the immiscible organic solution very slowly into an aqueous phase through a narrow needle at a temperature that the organic solvent is removed by vaporization during the process. In this method, large vesicles are formed which might be

due to the slow vaporization of solvent giving rise to an ether: water gradient extending on both sides of the interfacial lipid monolayer, resulting in the eventual formation of a bilayer sheet which folds in on to itself to form a sealed vesicle [29]. Ether injection treats sensitive lipids very gently and runs very little risk of causing oxidative degradation. Since the solvent is removed at the same rate as it is introduced, there is no limit to the final concentration of lipid which can be achieved, since the process can be run continuously for a long period of time, giving rise to a high percentage of the aqueous medium encapsulated within the liposomes. The major drawbacks of this method are the long time taken to produce a batch of liposomes and the need of careful control for the introduction of lipid solution.

2.4. Surfactant (detergent) solubilization method

In this method, the phospholipids are brought into contact with the aqueous phase via the intermediary of surfactants. Phospholipid molecules associate with surfactants and form mixed micelles. The basic feature of this method is the removal of the surfactant from pre-formed mixed micelles containing phospholipids, whereupon unilamellar liposomes form spontaneously. However, removal of surfactants is carried out using techniques such as, dialysis and column chromatography, inevitably remove other small water-soluble molecules, making this method not very efficient in terms of percentage encapsulation values attainable for water soluble compounds. On the other hand, surfactant solubilization method has the ability to vary the size of the liposomes by precise control of the conditions of surfactant removal and to obtain liposomes of very high size homogeneity [29].

The transfer from laboratory to industry was very important for liposomes, as it is for any biotechnological discipline. The first liposomal drug delivery experiments in humans were carried out by freshly prepared liposomes but in order to be a commercial product the liposome-drug formulation must have well-defined stability and a shelf life over a year. Of the several preparation methods described in the literature, only a few of them have the potential to be used in the large scale liposome manufacturing. The crucial problem is the presence of organic solvent residues, pyrogen control, stability, sterility, size and size distribution as well as batch to batch reproducibility.

In the parental administration the liposomes two important conditions involve being sterile and pyrogen free. In the case of animal experiments, the sufficient sterility can be obtained by the passage of the liposome preparations through the 400 nm pore size Millipore filters. In human experiments the sterilization depyrogenation techniques should be taken much more seriously starting from the raw materials, containers and working areas [41].

2.5. Loading of drugs in liposome formulations

2.5.1. Encapsulation of hydrophilic drugs

Once lipids are hydrated in the presence of hydrophilic drugs, a portion of the drug gets entrapped inside the liposome and another portion remains in the bulk, outside the aqueous core of the liposome. As only the entrapped drug is of interest, drug in the bulk should be

removed. This purification is generally done by gel filtration column chromatography (Sephadex G-50, Pharmacia LKB) and dialysis (hollow fiber dialysis cartridge) on the basis of size differences between the liposomes and the non-encapsulated material. In the cases where DNA or proteins are being encapsulated, or where there is concern that non-encapsulated material may form large aggregates, techniques such as centrifugation can be employed due to the differences in the buoyant densities of liposomes and non-encapsulated material [42, 43].

A hydrophilic drug may not be encapsulated with high efficiency because the drug molecules can diffuse in and out of the lipid membrane. Thus, the drug would be difficult to retain inside the liposomes. However, compounds with ionizable groups and those that are both water and lipid soluble can be encapsulated with high efficiency (up to 90%) by the liposomes after the formation of membranes [44] by active loading. In this technique, the pH of the interior part of the liposome is such that the unionized drug which enters the liposome by passive loading is ionized inside the liposome, and ionized drug molecules lose their ability to diffuse through the lipid membrane. Therefore, high concentration of the ionized drug is obtained inside the liposome. For example, doxorubicin and epirubicin can be entrapped in preformed SUV with high efficiency through active loading [45, 46].

The pH difference can be brought about by encapsulating a non-permeating buffer ion such as glutamate inside the liposomes at low pH and replacing the extra-liposomal buffer with one which is iso-osmolar at pH 7.0. Alternatively, charged lipids may be incorporated into the membrane at low pH, followed by adjustment of the suspending medium to neutrality. A similar approach may be adopted by using a potassium gradient, in which the membrane is made selectively permeable to potassium ions entrapped inside the liposome by incorporation of valinomycin into the lipid membrane [47, 48].

2.5.2. Encapsulation of hydrophobic drugs

Hydrophobic drugs are solubilized in the phospholipid bilayer of the liposomes that mainly provide a hydrophobic environment. Once trapped, they remain in the liposome bilayer as they have very low affinity towards the inner or outer aqueous regions of the liposomes. During the preparation of liposomes, hydrophobic drugs are solubilized in the organic solvent along with the phospholipids and during the subsequent hydration phase, they remain entrapped in the hydrophobic bilayer region. For example, the liposomal photosensitizer verteporfin (Visudyne) contains a hydrophobic drug that is rapidly transferred to blood proteins *in vivo*. Activation of the drug by targeting laser light to blood flowing through the eye causes its site-specific activity in the treatment of wet macular degeneration [49]. Amphotericin B and paclitaxel are the other most commonly investigated hydrophobic drugs in liposome formulations.

3. Stability of liposomes

Liposome stability can be explained by physical, chemical and biological means which are all interrelated. Generally, chemical (degradation of phospholipids structures) and physical

(uniformity of size distribution and encapsulation efficiency) stability determine the shelf-life of liposomes. Once the liposomal formulations have been obtained, maintenance of the physical properties of these preparations can be difficult. Leakage of the encapsulated material due to the permeability of the membrane, change in the size distribution and stability problems due to the hydrolytic and oxidative degradation are the general problems upon storage. Methods are devised to overcome these instability problems, those designed to minimize the degradation processes and those which help liposomes to survive in the face of conditions which encourages these processes.

Two different types of chemical degradation can affect the performance of the phospholipids bilayers; hydrolysis of the ester bonds linking the fatty acids to the glycerol backbone and oxidation of the unsaturated acyl chains, if present. The level of oxidation can be kept to a minimum by taking some precautions like starting with freshly purified lipids and freshly distilled solvents, avoiding procedures involving high temperatures, carrying out the manufacturing process in the absence of oxygen, deoxygenating the aqueous solutions by passing nitrogen, storing all liposome suspensions in an inert atmosphere and including an anti-oxidant, e.g. α -tocopherol [50], a common non-toxic dietary lipid, as a component of the lipids membrane. An alternative solution to the oxidation problem is to reduce the level of oxidizable lipids in the membrane by using saturated lipids instead of the unsaturated ones. Also, the mono-unsaturated ones have much less tendency of oxidation than the polyunsaturated ones. Thus; sphingomyelins, usually having only one double bond, are expected to degrade more slowly than other mammalian origin lipids. Entirely synthetic and saturated phospholipids; DMPC, DPPC and DSPC, can also be considered as a solution for the oxidative degradation of liposomes.

Hydrolysis type of chemical degradation of the ester linkages in the phospholipid structure occurs most slowly at pH values close to neutral. In general, the rate of hydrolysis has a "V-shaped" dependence, with a minimum at pH 6.5 and an increased rate at both higher and lower pH. In the active loading of drugs, as it is mentioned before, low pH levels are required which triggers the hydrolysis. This hydrolysis kind of chemical degradation is also very effective on the aqueous solutions of liposome due to the presence of water. Temperature also triggers the hydrolysis of the lipids which creates the need for refrigeration. In order to keep hydrolysis to a minimum during active loading, attention must be paid for the removal of residual solvent from the dried lipids. To avoid hydrolysis, instead of ester linked lipids, the usage of ether linkage containing lipids (e.g. found in the membrane of halophilic bacteria) would be an absolute solution [51]. Another chemical degradation, oxidation of the lipids in the liposome structures can be prevented by the addition of small amounts of antioxidants during the manufacturing steps.

The problems related to the lipid oxidation and hydrolysis during the shelf-life of the liposomal product can be reduced by the storage of liposomal dispersion in the dry state by freeze-drying (lyophilization), without compromising their physical state or encapsulation capacity [52]. However, freeze-drying of liposome systems without appropriate stabilizers will lead to fusion of vesicles, i.e. physical instability. To promote vesicle stability during the freeze-drying process, cycloprotectants [53-55], including saccharides (e.g. sucrose, trehalose, and lactose)

and their derivatives are employed [56]. Cycloprotectants, especially sucrose because of its high glass transition temperature, are believed to be effective to protect the liposome membranes against possible fracture and rupture that might cause a change in size distribution and a loss of the encapsulated material presumably by forming glasses under the typical freezing conditions used for lyophilization [57]. Lyophilization increases the shelf-life of the finished product by preserving in a relatively more stable dry state. Some liposome products on market or clinical trials are provided as lyophilized powder. For example, AmBisome™, a liposomal amphotericin, is the first liposome product to be marketed in several countries is supplied as a lyophilized powder to be reconstituted with sterile water injection. Additionally, paclitaxel-liposome formulations have been developed which show good stability [58, 59]. These formulations once lyophilized can be stored at room temperature for extended time. On the other hand, once the preparation is reconstituted, it is not stable for more than a day in terms of size.

The physical degradation, leakage and fusion of liposomes, can occur as a result of the lattice defects in the membrane introduced during the manufacture, particularly in SUVs that are prepared below the membrane phase transition temperature. Annealing process, incubating the liposomes at a higher temperature than the phase transition temperature, can wipe out these defects by equalizing the differences in packing density between opposite sides of the bilayers. Even in annealed vesicles, aggregation and fusion can occur over a long period of time. In neutral liposomes, aggregation takes place because of the van der Waals interactions and because of the increased surface area it tends to be more pronounced in large liposomes. The simplest solution to overcome this aggregation is to add a small amount of negatively charged phospholipid (e.g. 10% PA or PG) to the liposome composition [29]

SUVs have much more tendency to fusion when compared to large liposomes due to the presence of stress arising from the high curvature of the membrane. Since this can occur specifically at the transition temperature of the membrane, it would be better to store these liposomes at a temperature much lower than the transition temperature of the lipids. For example, SUVs should be stored above their transition temperature for no longer than 24 hours but LUVs can be stored for a longer period of time if the temperature of the solution is kept in a range of 4-8 °C for approximately 1 week before the leakage of the encapsulated material starts due to the hydrolytic degradation on the membrane structure [60]. Also, addition of cholesterol to the phospholipid mixture would be a solution to reduce or eliminate the transition. The presence of cholesterol prevents packing and aggregation by inducing orientation and more rigidity to the phospholipids. Other than cholesterol, peptide incorporation to the lipid membrane also enables the lipid membrane to be more rigid at physiological temperature [61-63].

Permeability of liposome membranes depends highly on the membrane lipid composition, as well as on the encapsulated material. Large polar or ionic molecules will be retained much more efficiently than low molecular weight lipophilic compounds. Generally, for both type of encapsulated material, a rigid, more saturated membrane with a higher ratio of cholesterol forms the most stable lipid membrane concerning the leakage of the encapsulated material.

Many attempts have been made to enhance the physical stability of liposomes. Among these, surface modification of liposomes is an attractive method to improve liposomal stability both *in vitro* and *in vivo*. Some improvements in chemical and physical stability of polymer coated liposomes prepared with polysaccharide derivatives, such as mannan or amylopectin, have been demonstrated [64]. Several other substances also have been used for preparation of polymer coated liposomes such as poloxamer, polysorbate 80, carboxymethyl chitosan, and dextran derivatives [65-69]. While the possibility of coating liposomes with these polymers has been reported, few papers have dealt with the systematic evaluation of the physical stability of polymer coated liposomes. Moreover, contravening results have been also reported such as that polymer coated liposomes showed less stability than non-coated ones [65, 70].

In vivo stability of liposomes is also dependent on their charge. In serum, there are several proteins that are both positively and negatively charged. Liposomes with neutral charge are found to be more stable as they have much less electrostatic affinity towards proteins. [71].

Biological liposome stability plays important roles at various stages of drug delivery. However, liposomes are somewhat biologically unstable as a parenteral drug delivery system owing to their rapid uptake and clearance from circulation by cells of the mononuclear phagocytic system (MPS) located mainly in the liver and spleen [72, 73]. Biological stability of liposomes is dependent on the presence of agents such as proteins that interact with liposomes upon application to the subject and the administration route. There have been many strategies to enhance the biological stability of liposomes that improve the liposomal drug delivery *in vivo* and increase the circulation time in blood stream [74]. The complexation between polymers and liposomes has been studied as a way to increase the long-term stability of liposomes. Grafting hydrophilic polymers onto the head groups of phospholipids, or the addition of water soluble polymers containing several hydrophobic groups has been shown to increase the circulation time *in vivo*, as well as to inhibit liposome fusion [75-77]. These kinds of liposomes are called stealth liposome [78] or sterically stabilized liposomes [79]. The steric repulsion of these liposomes stabilizes the liposomes against aggregation. One of the most popular and successful methods to obtain long-circulating biologically stable liposomes is to coat the surface of the liposome with poly(ethylene glycol), PEG [80-84]. Although the PEG chemistry is successful in coating the liposome surface, alternative sterically protecting polymers are also under research. The candidate polymers should be biocompatible, soluble, hydrophilic and have highly flexible main chain for drug delivery. Some of these polymers given in the literature are synthetic polymers of vinyl series i.e. poly(vinyl pyrrolidone) (PVP) and poly(acrylamide) (PAA) [85, 86]. PVP has a similar history on pharmaceutical application to PEG [87, 88]. It shows high degree of biocompatibility and also acts as efficient steric protector for liposomes. It was found that the liposomal bilayers containing lipids with covalently attached to polyethylene glycol by which the membrane surface sterically inhibits protein and cellular interactions with liposomes drastically prolonging the blood circulation time when injected in animals [89]. Doxil® is the liposomal doxorubicin available in the market which is stable for more than 18 months in the liquid state due to being stabilized by the usage of polyethylene glycol.

4. Sterilization of liposomes

Pharmaceutical industry in general differentiates between two principally different approaches to ensure sterility of a parental product: terminal sterilization of the final product in its container (steam sterilization) and aseptical manufacturing. Terminal sterilization is the commonly used one because of its higher sterility assurance level achieved when compared with the aseptical methods. However, terminal sterilization is not applicable to many liposomal drug carrier formulations.

There are several sterilization methods; such as filtration, gamma irradiation, final steam sterilization, dry heat sterilization, ethylene oxide sterilization, and ultraviolet sterilization. Bearing in mind the susceptibility of liposomes to the previously mentioned physical and chemical degradation mechanisms, the conditions required in conventional sterilization techniques (except filtration) are rather concerning since they involve the usage of heat, radiation and/or chemical sterilizing agents. Therefore, identification of a suitable method for sterilization of liposome formulations is a major challenge.

Sterilization Technique	Advantage(s)	Disadvantage(s)	Convenience
Filtration	Low operation temperature	Applicable to liposomes lower than 200 nm in diameter Operation under aseptic conditions	Low
γ -irradiation	Moderate operation temperature Highest microbial death reliability	Large scale operation Risk of degradation of liposomes	High
Final steam sterilization	Low cost and convenient	Risk of degradation of liposomes	High
Dry heat	Low cost and convenient	Risk of degradation of liposomes	High
Ethylene oxide	Low operation temperature	Possible carcinogenic residues	Low
UV-sterilization	Low cost and convenient	Poor penetration into products Risk of degradation of liposomes	High

Table 1. Summary of the Sterilization Techniques Applied on Liposomal Preparations.

Filtration is the most suitable sterilization technique for the thermolabile liposomes since it does not include any form of heat or condition that can result in the degradation of liposomes or leakage of the encapsulated material. However, filtration has some drawbacks such as; being only applicable to the liposomes that are smaller than 200 nm in diameter and being an expensive method due to the equipment requiring to work under high pressure (25 kg/cm² and above). Additionally, this technique must be performed under aseptic conditions [90].

Filtration sterilization is relatively time-consuming and not efficient for the removal of viruses [91]. Studies have shown that polycarbonate membranes are less effective than hydrophobic Fluoropore membrane and cellulose acetate/surfactant-free membrane filtration units [91]. Although the limitations of filtration provoked researches on other sterilization methods, all resulted in the formation of degradation products via the previously mentioned degradation pathways. Filtration and the other methods are summarized according to their applicability on liposomal preparations in Table 1 [92], given above.

5. Characterization of liposomes

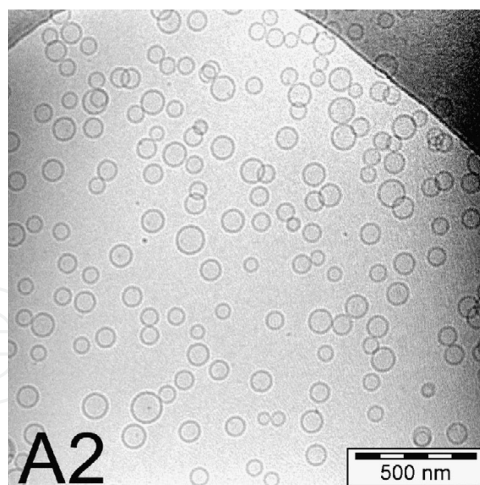
After preparation and before application, liposomes have to be characterized in order to ensure their *in vitro* and *in vivo* performance. Liposomal properties that are commonly discussed include lamellarity (the number of bilayers present in liposomes), diameter and size distribution, lipid composition and concentration determination, the encapsulant concentration and its encapsulation efficiency.

For the characterization of chemical properties, phospholipids can be quantitatively in terms of concentration either by Bartlett Assay or Stewart Assay. The phospholipid hydrolysis might be followed by HPLC where the column outflow can be monitored continuously by UV absorbance to obtain a quantitative record of the eluted components. Moreover, the phospholipid oxidation can also be followed by a number of techniques *i.e.*, UV absorbance method, TBA method (2-thiobarbutiric acid) (for endoperoxides), iodometric method (for hydroperoxides) and GLC (gas-liquid chromatography) method [93].

The most direct method for determination of liposome size is the electron microscopy due to the possibility of viewing the liposomes individually and obtaining the exact information about the liposome population over the whole range of sizes [94]. As liposomes do not naturally create a contrast to be visible by electron microscopy, either cryo-TEM (Figure 2) should be used or staining of the liposome sample is required. Either way, it is a very time-consuming method and it requires equipments that may not always be immediately accessible. The other method for the determination of liposome size, dynamic light scattering [95, 96], is very simple and rapid to perform but it measures an average size of liposome bulk. More recently, atomic force microscopy is also used to determine the morphology, size and stability of liposomal structures. All these size determination methods are very expensive. If only an approximate size range is required, gel exclusion chromatography might be suitable.

Electrostatic stabilization of liposomes may be a desirable feature to prevent fusion. The surface charge on the liposomes is measured by zeta-potential measurements [98]. These measurements are useful in determination of the *in vivo* behavior of liposomes. Often zeta potential values <-25 mV or $>+25$ mV are considered stable [99]. However, as mentioned earlier, charged liposomes have the disadvantage of being unstable in biological conditions.

Residual solvent is very unacceptable for drug delivery applications, therefore residual solvent should be kept at a minimum in the formulations. Quantification of residual solvents as a result



Adopted from Holzer, M., Barnert, S., Momm, J., Schubert, R., 2009. Preparative size exclusion chromatography combined with detergent removal as a versatile tool to prepare unilamellar and spherical liposomes of highly uniform size distribution. *J. Chromatogr. A* 1216, 5838–5848.

Figure 2. Cryo-TEM pictures of Size Exclusion Chromatography fractions eluted at 90 minutes and prepared from egg-phosphatidylcholine.

of preparation methods is done through gas chromatography (GC) [100-101] This is a very rapid and reliable method and most analytical and organic laboratories are equipped with a GC.

An important feature of liposomes is the existence of a temperature dependant, reversible phase transition, where the hydrocarbon chains of the phospholipid structures undergo a transformation from an ordered gel state to a more disordered fluid, liquid crystalline, state. This transition temperature is important in optimizing the storage conditions (i.e Temperature) to minimize fusion and drug leakage. These changes have been monitored by freeze fracture electron microscopy and much more easily by differential scanning calorimetry (DSC) [102-104, 93].

Entrapped volume is a crucial parameter that governs the morphology of liposomes. This internal volume is defined as the aqueous entrapped volume per unit quantity of lipids. The most promising way to determine the internal volume is to measure the quantity of water by replacing external medium (water) with a spectrophotometrically inert fluid (i.e. deuterium oxide) and then measuring water signal by NMR [93].

It is essential to measure the quantity of the encapsulated material inside liposomal structures before studying the behavior of this encapsulated material physically and biologically since the effects observed experimentally will be dose related. After the removal of the non-encapsulated material by the separation techniques the quantity of material remained can be assumed as 100% encapsulated. Minicolumn centrifugation and protamine aggregation methods are the general separation procedures that are commonly used [93].

Methods for determining the amount of material encapsulated within the liposomes typically rely on the destruction of the lipid bilayer and subsequent quantification of the released material [105]. In these measurements, the signal due to intact liposomes is typically monitored

prior to bilayer disruption. The techniques used for this quantification depend on the nature of the encapsulant and include spectrophotometry [106, 107], fluorescence spectroscopy [108], enzyme-based methods [109] and electrochemical techniques. If a separation technique such as HPLC or field-flow fractionation (FFF) is applied, the percent encapsulation can be expressed as the ratio of the unencapsulated peak area to that of a reference standard of the same initial concentration [110, 111]. This method can be applied if the liposomes do not undergo any purification following preparation. Either technique serves to separate liposome encapsulated materials from those that remain in the extravesicular solution and hence can also be used to monitor the storage stability in terms of leakage or the effect of various disruptive conditions on the retention of encapsulants. Some authors have combined the size distribution and encapsulation efficiency determination in one assay by using FFF-MALS (multi angled light scattering) coupled to a concentration detector suitable for the encapsulant [112].

Since techniques used to separate free materials from liposome-encapsulated contents can potentially cause leakage of contents and, in some cases, ambiguity in the extent of separation, research using methods that do not rely on separation are of interest. Reported methods have included ¹H NMR where free markers exhibited pH sensitive resonance shifts in the external medium versus encapsulated markers [113]; diffusion ordered 2D NMR which relied on differences in diffusion coefficients of entrapped and free marker molecules [114]; fluorescence methods where the signal from unencapsulated fluorophores was quenched by substances present in the external solution [115]; electron spin resonance (ESR) methods which rely on the signal broadening of unencapsulated markers by the addition of a membrane-impermeable agent [116, 117].

The drug release from liposomes can be followed by the usage of a well calibrated in vitro diffusion cell in order to predict pharmacokinetics and bioavailability of drug before expensive and time-consuming in vivo studies. For the determination of pharmacokinetic performance of liposomal formulations, dilution-induced drug release in buffer and plasma was employed and for the determination of drug bioavailability, another procedure is followed which involves the liposome degradation in the presence of mouse-liver lysosome lysate [93].

6. Clinical applications of liposomes

New drug delivery systems such as liposomes are developed when the existing formulations are not satisfactory. Among all the nanomedicine platforms, liposomes have demonstrated one of the most established nanoplatfroms with several FDA-approved formulations for cancer treatment, and had the greatest impact on oncology to date, because of their size, biocompatibility, biodegradability, hydrophobic and hydrophilic character, low toxicity and immunogenicity [118]. A vast of literature describes the feasibility of encapsulation of a wide range of drugs, including anti-cancer and antimicrobial agents, peptide hormones, enzymes, other proteins, vaccines and genetic materials, in the aqueous or lipid phases of liposomes which showed enhanced therapeutic activity and/or reduced toxicity in preclinical models and in humans when compared to their non-liposomal formulations.

Liposome applications in drug delivery depend, and are based on, physicochemical and colloidal characteristics such as composition, size, loading efficiency and the stability of the

carrier, as well as their biological interactions between liposomes and cells. Based on these liposome properties, several modes of drug delivery can be listed: the major ones are enhanced drug solubilization (e.g. amphotericin B, minoxidil), protection of sensitive drug molecules (e.g. cytosine arabinose, DNA, RNA, antisense oligonucleotides, ribozymes), enhanced intracellular uptake (all agents, including antineoplastic agents, antibiotics and antivirals) and altered pharmacokinetics and biodistribution of the encapsulated drug.

Although lipid based formulations have advantages as drug carriers, drug-delivery systems based on unmodified liposomes are limited by their short blood circulation time, instability in vivo and lack of target selectivity [119, 120]. To increase accumulation of liposomal formulations in the desired cells and tissues, the use of targeted liposomes including surface-attached ligands such as; antibodies, folates, peptides and transferrin that are capable of recognizing and binding to the desired cells. Despite of some improvements in targeting efficiency by these immunoliposomes, the majority of these modified liposomes were still eliminated rapidly by the reticulo endothelial system, primarily in the liver [120]. Better target accumulations are expected if liposomes can be made to remain in the circulation long enough.

Schematic drawing of cytosolic delivery and organelle-specific targeting of drug loaded nanoparticles (i.e. most frequently liposomes) via receptor-mediated endocytosis is shown in Figure 3.

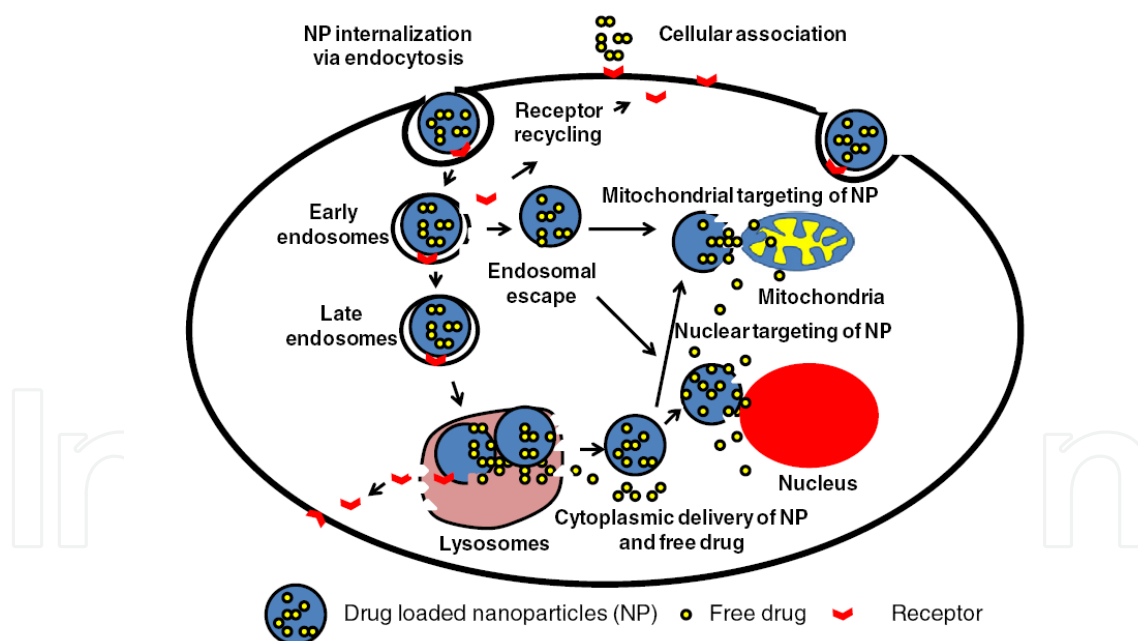


Figure 3. Schematic drawing of the cytosolic delivery and organelle-specific targeting of drug loaded nanoparticles via receptor-mediated endocytosis. After receptor mediated cell association with nanoparticles, the nanoparticles are engulfed in a vesicle known as an early endosome. Nanoparticles formulated with an endosome disrupting property disrupt the endosomes followed by cytoplasmic delivery. On the other hand, if nanoparticles are captured in early endosomes, they may make their way to lysosomes as late endosomes where their degradation takes place. Only fraction of non-degraded drug released in the cytoplasm interacts with cellular organelles in a random fashion. However, cytosolic delivery of a fraction of organelle-targeted nanoparticles via endosomal escape or from lysosomes travel to the targeting organelles to deliver their therapeutic cargo [121].

Different methods have been suggested to achieve liposomes with high stability and long circulation times in vivo, including the surface coating of the liposomes with inert, biocompatible polymers such as PEG (stealth liposomes), which forms a protective layer over the liposome surface and slow down liposome recognition by opsonins and therefore subsequent clearance of liposomes [80, 84]. Long circulating liposomes are now being investigated in detail and are widely used in vitro and in vivo studies due their flexibility and also they found their place in the clinical applications. The flexibility allows a relatively small number of surface-grafted polymer molecules to create an impermeable layer over the liposome surface [122, 123]. Long-circulating liposomes demonstrate dose-dependent, non-saturable, log-linear kinetics and increased bioavailability [124].

The studies that attempt to combine the properties of long-circulating liposomes and immunoliposomes in one preparation place themselves in the literature as the further development in the liposomal formulations as drug carriers [125, 126]. In the early experiments, simple co-immobilization of an antibody and PEG on the surface of the same liposome has been performed despite the possibility of PEG creating steric hindrance for target recognition with the targeting moiety [125]. To achieve better selectivity of PEG-coated liposomes, it is advantageous to attach the targeting ligand via a PEG spacer arm, so that the ligand is extended outside the dense PEG brush which reduces steric hindrance of binding to the target [127]. The use of PEG-conjugated immunoliposomes for increasing drug carrying capacity of monoclonal antibody has been demonstrated [128]. In addition to costly monoclonal antibodies, common molecules such as folic acid, transferrin and RGD peptides have also been studied for tumor targeting with enhanced selective uptakes [120].

Encouraging results of liposomal drugs in the treatment or prevention of a wide spectrum of diseases in experimental animals and in human, indicate that more liposome-based products for clinical and veterinary applications may be forthcoming. These could include treatment of eye and skin diseases in therapeutic applications, antimicrobial and anticancer therapy in clinical applications, metal chelation, enzyme and hormone replacement therapy, vaccine and diagnostic imaging, etc. Some of the liposome applications in terms of drug delivery are discussed below.

6.1. Ocular applications

The eye is protected by three highly efficient mechanisms (a) an epithelial layer which is the barrier to penetration (b) tear flow (c) the blinking reflex. All these mechanisms are responsible for the poor drug penetration into the deeper layers of the cornea and the aqueous humor and for the rapid wash out of drugs from the corneal surface. Initially, in 1981 the enhanced efficiency of liposomes encapsulated idoxuridine in herpes simplex infected corneal lesions in rabbits was reported [129]. In 1985, it was concluded that ocular delivery of drugs can be either promoted or impeded by the use of liposome carriers, depending on the physicochemical properties of the drugs and the lipid mixture employed [130]. The use of mucoadhesive polymers, carbopol 934P and carbopol 1342 to retain liposomes at the cornea was proposed [131]. While precorneal retention times were indeed significantly enhanced under appropriate

conditions, liposomes even in the presence of the mucoadhesive had migrated toward the conjunctival sac with very little activity remaining at the corneal surface.

6.2. Pulmonary applications

Lung is a natural target for the delivery of therapeutic and prophylactic agents such as peptides and proteins. The past 15 years have been marked by intensive research efforts on pulmonary drug delivery not only for local therapy but also for systemic therapy as well as diagnostic purposes, primarily due to the several advantages the pulmonary route offers over other routes of drug administration. Drugs that undergo gastrointestinal degradation (such as proteins and peptides) are ideal candidates for pulmonary delivery.

Targeted drug delivery to the lungs has evolved to be one of the most widely investigated systemic or local drug delivery approaches. The use of drug delivery systems for the treatment of pulmonary diseases is increasing because of their potential for localized topical therapy in the lungs. This route also makes it possible to deposit drugs more site-specific at high concentrations within the diseased lung thereby reducing the overall amount of drug activity while reducing systemic side effects. To further exploit the other advantages presented by the lungs, as well as to overcome some challenges, scientists developed interests in particulate drug delivery systems for pulmonary administration, such as liposomes, micelles, nano- and micro-particles based on polymers.

The use of liposomes as drug carriers for pulmonary delivery has been reported for different kinds of therapeutics such as anti-microbial agents, cytotoxic drugs, antioxidants, anti-asthma compounds and recombinant genes for gene therapy in the treatment of cystic fibrosis.

Liposomes as carrier systems for pulmonary delivery offer several advantages over aerosol delivery of the corresponding non-encapsulated drug. Liposomes might be used to solubilize poorly soluble drugs, provide a pulmonary sustained release reservoir prolonging local and systemic therapeutic drug levels, facilitate intracellular delivery of drugs especially to alveolar macrophages, tumor cells or epithelial cells, prevent local irritation of lung tissue and reduce the drug's toxicity, target specific cell populations using surface bound ligands or antibodies and be absorbed across the epithelium to reach the systemic circulation intact [132].

Local delivery of medication to the lungs is highly desirable, especially in patients with specific pulmonary diseases such as cystic fibrosis, asthma, chronic pulmonary infections or lung cancer. The principal advantages include reduction of systemic side effects and application of higher doses of the medication at the site of drug action. Although simple inhalation devices and aerosols containing various drugs have been used since the early 19th century for the treatment of respiratory disorders, the past 15 years have been marked by intensive research efforts on pulmonary drug delivery not only for local therapy but also for systemic therapy as well as diagnostic purposes due to the several advantages the pulmonary route offers over other routes of drug administration. Lung is a natural target for the delivery of therapeutic and prophylactic agents such as peptides and proteins due to the large surface area available for absorption, the very thin absorption membrane and the elevated blood flow which rapidly distributes molecules throughout the body. Moreover, the lungs exhibit relatively low local

metabolic activity, and unlike the oral route of drug administration, pulmonary inhalation is not subject to first pass metabolism [133].

Inhaled drug delivery devices can be divided into three principal categories: nebulizers, pressurized metered-dose inhalers and dry powder inhalers; each class presents unique strengths and weaknesses. A good delivery device has to generate an aerosol of suitable size and provide reproducible drug dosing. It must also protect the physical and chemical stability of the drug formulation.

For controlled delivery of drug to the lung, liposomes are one of the most extensively investigated systems in recent studies given that they can be prepared with phospholipids such as egg phosphatidylcholine (PC), distearoyl phosphatidylcholine (DSPC) and dipalmitoylphosphatidylcholine (DPPC) endogenous to the lung.

A significant disadvantage of many existing inhaled drugs is the relatively short duration of resultant clinical effects, which requires most medications to be inhaled at least twice daily. This often leads to poor patient compliance. A reduction in the frequency of dosing would be convenient, particularly for chronic diseases such as asthma. The advantages of such an approach include reduced dosing, increased effectiveness of rapidly cleared medicine and enhanced residence time at the target site for the treatment of infection. Many challenges exist in developing controlled release inhalation medicine, which is reflected in the fact that no commercial product exists. Cytotoxic agents, bronchodilators, anti-asthma drugs, antimicrobial and antiviral agents and drugs for systemic action, such as insulin and proteins are being investigated.

6.3. Cancer therapy

The numerous anti-cancer agents that have a high cytotoxic effect on the tumor cells in vitro exhibit a remarkable decrease of the selective anti-tumor effect for in vivo procedures applicable in the clinical treatment. One of the significant limitations of the anti-cancer drugs is their low therapeutic index meaning that the dose required to produce an anti-tumor effect is toxic to normal tissues. The low therapeutic index of these drugs results from the inability to achieve therapeutic concentrations at the specific target sites, tumors. Further, it results from the non-specific toxicity to normal tissues such as bone marrow, renal, gastrointestinal tract, and cardiac tissue and also from the problems associated with a preparation of a suitable formulation of the drugs [134].

Many different liposome formulations of various anticancer agents were shown to be less toxic than the free drug so that most of the medical applications of liposomes that have reached the preclinical stage are in cancer treatment [135-137]. Entrapment of these drugs into liposomes resulted in increased circulation lifetime, enhanced deposition in the infected tissues, and protection from the drug metabolic degradation, altered tissue distribution of the drug, with its enhanced uptake in organs rich in mononuclear phagocytic cells (liver, spleen and bone marrow) and decreased uptake in the kidney, myocardium and brain. To target tumors, liposomes must be capable of leaving the blood and accessing the tumor. However, because of their size liposomes cannot normally undergo transcapillary passage. In spite of this, various

studies have demonstrated the accumulation of liposomes in certain tumors in a higher concentration than found in normal tissues [138, 139]. Anthracyclines are drugs which stop the growth of dividing cells by intercalating into the DNA and therefore kill predominantly quickly dividing cells. These cells are not only in tumors but are also in hair, gastrointestinal mucosa, and blood cells; therefore, this class of drugs is very toxic. Many research efforts have been directed towards improving the safety profile of the anthracyclines cytotoxics, doxorubicin and daunorubicin, along with vincristine. Encapsulation of these drugs into the liposomes showed reduced cardiotoxicity, dermal toxicity and better survival of the experimental animals compared to the controls receiving free drugs [138]. Such beneficial effects of liposomal anthracyclines have been observed with a variety of liposome formulations regardless of their lipid composition and provided that lipids used high cholesterol concentration of phospholipids with high phase transition temperature are conducive to drug retention by the vesicles in the systemic circulation [45].

Active targeting of cancer drugs to the tumors is shown schematically in Figure 4.

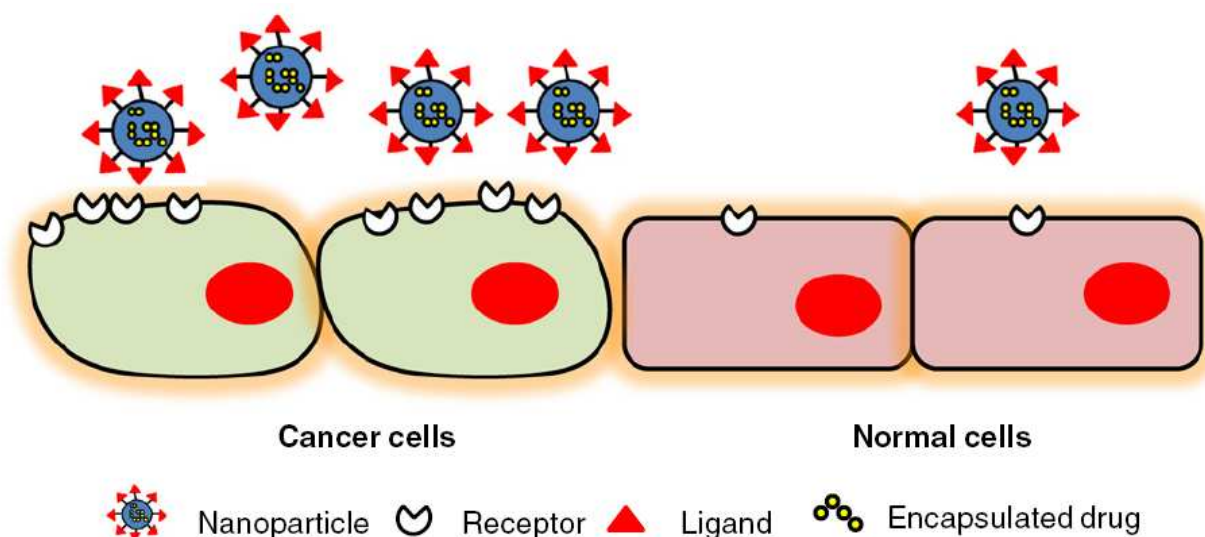


Figure 4. Representation of active targeting via receptors [121].

Currently several liposomal formulations are in the clinical practice containing different chemotherapeutics such as doxorubicin (Doxil/Caelyx1), doxorubicin (Myocet1), daunorubicin (DaunoXome1) and cytarabine (DepoCytel) for treating the ovarian cancer, AIDS related Kaposi's sarcoma, multiple myeloma, lymphomas, leukemia with meningeal spread. Several other liposomal chemotherapeutic drugs containing doxorubicin, annamycin, mitoxantrone, cisplatin, oxaliplatin, camptothecin, 9-nitro-20 (S)-camptothecin, irinotecan, lurtotecan, topotecan, paclitaxel, vincristine, vinorelbine and floxuridine are at the various stages of clinical trials [140].

Two liposomal formulations have been approved by the US Food and Drug Administration (FDA) and are commercially available for the treatment of AIDS-related Kaposi's sarcoma. Doxil, first liposomal drug approved by FDA and has been on the market since 1995, is a

formulation of doxorubicin precipitated in sterically stabilized liposomes and has been on the market since 1995 [141], while DaunoXome, approved six months later than Doxil, is daunorubicin encapsulated in small liposomes with very strong and cohesive bilayers, which can be referred as mechanical stabilization [142].

DaunoXome is composed of small unilamellar vesicles containing distearoylphosphatidylcholine-cholesterol (2:1) with daunorubicin loaded by a pH gradient [137]. These liposomes are selectively stable in the circulation because they are small and their membrane is electrically neutral and mechanically very strong [142]. This reduces the charge-induced and hydrophobic binding of plasma components but does not protect against van der Waals adsorption. Also, uncharged liposomes are colloiddally less stable than charged ones.

Doxil is a liquid suspension of 80-100 nm liposomes (2000PEG-distearoylphosphatidylethanolamine-hydrogenated-soya-bean phosphatidylcholine-cholesterol, 20 mM) loaded with doxorubicin HCl by ammonium sulfate gradient technique and additionally precipitation with encapsulated sulfate anions. These liposomes circulate in patients for several days, which increase their chances of extravasating at sites with a leaky vascular system. Their stability is due to their surface PEG coating as well as to their mechanically very stable bilayers [141, 142].

Cytarabine (Ara-C) is an effective hydrophilic chemotherapeutic agent used widely for the treatment of acute myelogenous leukaemia and lymphocytic leukaemia [143]. It has often been utilized in the combination chemotherapy, against solid tumors and leukaemias. Cytarabine is a cell cycle-dependent drug; hence, prolonged exposure of cells to cytotoxic concentrations is critical to achieve maximum cytotoxic activity. The toxicity of cytarabine is reduced if it is able to maintain an effective therapeutic level for a long period of time and, thus, it is a suitable candidate for administration in a controlled-release dosage form. Liposome encapsulated liposomes (DepoCyt™) are now commercially available.

Etoposide (VP-16-213) is another successful chemotherapeutic agents used for the treatment of human cancers. The drug is currently in its third decade of clinical use and is a front line therapy for a variety of malignancies, including leukaemias, lymphomas and several solid tumors [144]. It has a short biological half-life (3.6 h) with a terminal half-life of 1.5 h intravenously and a variable oral bioavailability ranging from 24% to 74%. Although intraperitoneal injection would result in initial high local tumor concentrations, prolonged exposure of tumor cells may not be possible [145].

The harmful and even destructive effect of cytotoxic drugs on healthy body cells makes it necessary to search for new delivery methods for drugs like cytarabine and etoposide. There are many articles describing the results of investigations of incorporation of cytarabine [146] and etoposide [147] into liposome. However, there is no information about their simultaneous incorporation, in spite of the fact that these two drugs have been used for more than 30 years.

Taxanes are complexes of diterpenoid natural products and semisynthetic analogs. Presently, these drugs belong to prominent anticancer agents used for combined chemotherapy [148]. Paclitaxel (PTX), the prototype of this class, emerges from a natural source [149]. This drug have been used for various cancers including ovarian, breast, head and neck, and non-small cell lung cancers [150].

The commercial PTX preparation (Taxol®) is formulated in the vehicle composed of Cremophor EL® (polyethoxylated castor oil used as a solubilizing surfactant) and dehydrated ethanol, which provides a homogenous preparation. However, some drawbacks have been reported for its clinical applications of this formulation such as severe hypersensitivity reactions, neurotoxicity and neutropenia [151, 152]. It was reported that these adverse effects associated with this formulation would be due to Cremophor EL rather than PTX itself [153]. PTX solubilized in Cremophor EL shows also an incompatibility with the polyvinyl chloride of the administration sets [152]. Furthermore, the short-term stability of PTX upon dilution with aqueous media can result in possible drug precipitation [154].

Special requirements regarding a proper filter device as well as appropriate containers and infusion bags for the storage and administration of the drug have to be fulfilled in order to overcome the problems of incompatibility and instability during the clinical application of Taxol®. Hence, the development of an improved delivery system for PTX is of high importance. Current approaches are focused mainly on the development of formulations that are devoid of Cremophor EL, investigation of the possibility of a large-scale preparation and a request for a longer-term stability. There are some promising possibilities to replace Taxol® by a less irritable preparation such as micelle formulations, water-soluble prodrug preparations, enzyme-activatable prodrug preparations conjugated with antibodies or albumin, parenteral emulsions, microspheres, cyclodextrins, and nanocrystals [155-162].

The preparation of an optimal PTX formulation requires important considerations such as the optimization of the liposomal composition, the balance of the PTX amount encapsulated in the liposomes and the stability of the prepared PTX liposomes during storage in aqueous media [163]. The main characteristics of PTX molecule are asymmetry, bulkiness, hydrophobicity, low solubility and tendency to crystallization in aqueous media. All these factors affect the final design and preparation of a suitable drug formulation.

Liposomes provide suitable environment enhancing the solubility of the hydrophobic nature PTX by associating the molecule within the membrane bilayers. Commonly prepared formulations of PTX with liposomes were able to encapsulate the highest achievable content of PTX, 3-4 mol% with stability for weeks to months whereas 4-5 mol% paclitaxel was stable in the time range of just several hours to a day, and 8% paclitaxel loading only resulted in 15 minutes of liposome stability. Generally, increasing the encapsulated amount of PTX causes a reduction in the stability of the liposomal-PTX formulation due to the crystallization of the drug molecule. Thereby, to achieve a high drug/lipid ratio while retaining the long-term physical-chemical stability, a freeze-drying method is employed to obtain a dry drug-lipid powder, which is rehydrated in an aqueous solution immediately before use [58]. The encapsulation of PTX into liposomes enhances the drug therapeutic efficacy, thus, the same therapeutic effect could be reached by a decreased PTX-dose. On the other hand, the maximum tolerated dose (MTD) of liposome-encapsulated PTX increased compared with the Taxol® [Straubinger, R.M. and S.V. Balasubramanian, Preparation and Characterization of Taxane-containing Liposomes, *Methods Enzymol.* 391 (2005) 97-117.]. [163].

Taxane liposomes have shown slower elimination, higher antitumor activity against various murine and human tumors and lower systemic toxic effect compared to Taxol® [58]. They

have also shown antitumor effect in Taxol-resistant tumor models [164]. Abraxane®, the only nonliposomal preparation of PTX, (albumin nanoparticle-based PTX preparation) and Lipusu® (liposomal PTX approved by State FDA of China) have entered the field of clinical applications. LEP-ETU (NeoPharm) and EndoTAG®-1 (Medigene) have reached the phase II of the clinical trials. Generally, liposomes and protein nanoparticles represent a promising approach to the optimization of PTX delivery. Their commercialization is at the doorstep of modern drug delivery market.

7. New generation liposomes

Liposomes made up of commonly used ester phospholipids such as phosphatidylcholine are referred as conventional liposomes. These structures are very attractive for encapsulation and drug delivery applications to entrap both hydrophilic and hydrophobic materials due to the presence of aqueous core part as well as the lipid bilayer. Up to this date, there are many formulations in the market and also in the clinical trials. However, none of them truly overcome their chemical and physical instability problems especially during the transfer to the site of action [120]. Various attempts like modification of the liposome surface with i.e. hydrophilic polyethylene glycol polymers, using cryoprotectants or incorporation of high amount of cholesterol into the bilayer have led to only limited success. Other than instability problems, liposomal drug vehicles show extensive leakage of water-soluble drugs during the passage through the gastrointestinal tract and they are heterogeneous in terms of size distribution. Therefore, scientists have been looking for new drug delivery formulations that could address these issues about liposomes, which lead to the so-called new generation of liposomes which will be summarized in this section.

Archaeosomes are liposomal formulations that are prepared with one or more lipids, mainly containing diether and/or tetraether linkages, found in archaeobacterial membrane [165]. These archaeobacterial lipids present unique features and higher stabilities to several conditions (high or low temperatures, high salinity, acidic media, anaerobic atmosphere, high pressure) over conventional liposomes [166]. The definition of archaeosomes also includes the use of synthetically derived lipids that have the properties of archaeobacterial ether lipids, that is, regularly branched phytanyl chains attached via ether bonds at sn-2,3 glycerol carbons [167]. The surprising stability of archaeosomes can be attributed to some properties brought by the archaeobacterial lipids' structure: (i) the ether linkages that are more stable than esters over a wide range of pH, and the branching methyl groups help both to reduce crystallization and permeability; (2) the stability towards oxidative degradation of these lipid membranes are provided by the fully saturated alkyl chains in the archaeobacterial lipids; (3) the unusual stereochemistry of the glycerol backbone ensures the resistance of the membrane to enzymatic attack; (4) the bipolar lipids span the membranes and enhance their stability properties [167, 168].

Archaeosomes can be prepared by using conventional procedures (hydration of a thin film followed by sonication or extrusion, detergent dialysis) at any temperature in the physiolog-

ical range or lower, thus making it possible to encapsulate thermally labile compounds. Additionally, they can be prepared and stored in the presence oxygen without any degradation. According to the clinical experiments, *in vivo* and *in vitro*, these new drug delivery vehicles are not toxic. Thus, the biocompatibility and better stability of archaeosomes in numerous conditions offer advantages over conventional liposomes for their usage in biotechnology including vaccine and drug/gene delivery [167]. Consequently, they can be considered as better carriers than conventional liposomes, especially for protein and peptide delivery due to their high stability. Li et al. showed the superiority of archaeosomes over conventional liposomes in their study in which they used insulin as a model peptide for its oral delivery [169].

Another development aiming to enhance tissue targeting is virosomes in which the liposome surface is modified with fusogenic viral envelope proteins [170]. Virosomes have been used for the intracellular delivery of drugs and DNA [171, 172] as well as the basis of the newly developed vaccines which are very effective in the delivery of protein antigens to the immune system [173]. As a result, a whole set of virosomes-based vaccines have been developed for human and animal use. Special attention has been paid to the delivery of influenza vaccine using virosomes containing the spike proteins of influenza virus. Virosome-based vaccines were found to be highly immunogenic and well tolerated in children. A similar approach was used to prepare virosomal hepatitis A vaccine that elicited high antibody titres after primary and booster vaccination of infants and young children which was also confirmed for the healthy adults and elderly patients [174-176]. In general, virosomes can provide an excellent opportunity for the efficient delivery of both various antigens and many drugs, including nucleic acids, cytotoxic drugs and toxoids [177, 178], although they might present certain problems associated with their stability, leakiness and immunogenicity.

Niosomes, exhibiting a similar behavior to liposomes, are the vesicles that are made up of nonionic surfactants (e.g. alkyl ethers and alkyl esters) and cholesterol. These structures are stable on their own and they increase the stability of the encapsulated drugs. No special conditions are needed for handling and storage of these surfactants. Niosomes improve the oral bioavailability of poorly absorbed drugs, and enhance skin penetration of drug. When compared with liposomes, their oral absorption is better due to the replacement of phospholipids with nonionic surfactants which are less susceptible to the action of bile salts, parenteral, as well as topical routes. These delivery systems are biodegradable, biocompatible and non-immunogenic. Niosomes improve the therapeutic performance of drug molecules by delaying the clearance from the circulation and protecting the drug from biological environment [179].

The transdermal delivery is one of the most important routes of drug administration. The main factor which limits the application of transdermal route for drug delivery is the permeation of drugs through the skin. Human skin has selective permeability for drugs. Lipophilic drugs can pass through the skin but the drugs which are hydrophilic in nature can not pass through. Water soluble drugs either show less or no permeation. To improve the permeation of drugs through the skin various mechanisms have been investigated, including use of chemical or physical enhancers, such as iontophoresis, sonophoresis, etc. Liposomes and niosomes are not

suitable for transdermal delivery due to poor skin permeability, breaking of the system, aggregation, drug leakage, and fusion of vesicles [180].

A new type of carrier system, suitable for transdermal delivery, called transfersome has been proposed for the delivery of proteins and peptides like insulin, bovin, serum albumin, vaccines, etc. These systems are soft and malleable carriers that offer noninvasive delivery of drug into or across the deeper skin layers and/or the systemic circulation [181]. Transfersomes improve the site specificity while providing the safety of the drug. Transfersomes are the lipid supra-molecular aggregates which make them very flexible. This flexibility as well as their good penetration ability causes them to be used in the effective delivery of non-steroidal anti-inflammatory agents like ibuprofen and diclofenac [182].

Alternatively, unlike classic liposomes [183, 184], that are known mainly to deliver drugs to the outer layers of skin, ethosomes can enhance permeation through the stratum corneum barrier [185-187]. Ethosomes, developed by Touitou in 1997, are the slight modification of well established drug carrier liposome, containing phospholipids, alcohol (ethanol or isopropyl alcohol) in relatively high concentration and water [188]. The size of these soft vesicles can vary from nanometers to microns [189-193]. The high concentration of ethanol makes the ethosomes unique. The ethanol in ethosomes causes disturbance in the skin lipid bilayer organization, hence when incorporated into a vesicle membrane, it enhances the vesicle's ability to penetrate the stratum corneum. Also, because of the high concentration of ethanol the lipid membrane is packed less tightly than conventional vesicles but has equivalent stability, allowing a more malleable structure and improves drug distribution ability in stratum corneum lipids. Ethosomes can be used for many purposes in drug delivery for the treatment of many diseases such as Minoxidil for baldness, testosterone as steroidal hormone, Trihexyphenidyl hydrochloride for Parkinson's disease, Zidovudine and Lamivudine as anti-HIV, Bacitracin as antibacterial, Erythromycin as antimicrobial, DNA for genetic disorders, Cannabidol in the treatment of rheumatoid arthritis and many others [190, 192, 194-201].

Novasomes are the modified forms of liposomes [202] or a type of niosomes prepared from the mixture of monoester of polyoxyethylene fatty acids, cholesterol and free fatty acids with the diameter of 0.1-1.0 microns. They consist of two to seven bilayer shells that surround an unstructured space occupied by a large amorphous core of hydrophilic or hydrophobic materials [203]. The inner amorphous core can be loaded up to 80-85% with a medical drug and the surfaces of novasomes can be positive, negative or neutral.

Novasomes offer several advantages to the owners of the product such as: Both hydrophilic and hydrophobic products can be incorporated in the same formulation, drugs showing interactions can be incorporated in between bilayers to prevent incompatibility, they can be made site specific due to their surface charge characteristics, they can deliver a large volume of active ingredient, thus also reducing the frequency of application, and they have the ability of adhering skin or hair shafts which makes novasomes applicable in the cosmetic formulations [204].

Novasomes have extensive utilization in fields of foods, cosmetics, personal care, chemical, agrochemical and pharmaceuticals. The technology enhances absorption rate via topical

delivery of pharmaceuticals and cosmeceuticals by utilizing non-phospholipid structures. Various FDA-regulated products such as human pharmaceuticals and vaccines can be developed by this technology [205, 206]. These nonionic vesicles composed of glyceryl dilaurate with cholesterol and polyoxyethylene-10-stearyl ether have been known to deliver greater amounts of cyclosporine into and through hairless mouse skin than phosphatidyl choline or ceramide based vesicles [206]. Among various liposomal formulations, novasomes appeared more effective when delivered under non-occluded conditions from a finite dose [206]. Various vaccines based on novasomes have been licensed for the immunization of fowl against Newcastle disease virus and avian rheovirus [135]. Some of the novasome-based vaccines against bacterial and viral infections have been developed such as small pox vaccine while still many are under development [207]. Novasomes inactivate viruses such as orthomyxoviruses, paramyxoviruses, coronaviruses and retroviruses, etc., by fusing with enveloped virus and that the nucleic acid of the virus denatures shortly after the fusion [208].

Although liposomes are like biomembranes, they are still foreign objects of the body. Therefore, liposomes are known by the mononuclear phagocytic system (MPS) after contact with plasma proteins. Accordingly, liposomes are cleared from the blood stream. For more than two decades, various PEG derivatives have been used to stabilize for increasing efficiency in drug or gene delivery. Most 'stabilized' liposomes, the so-called stealth liposomes [78], or cryptosomes [84], contain a certain percentage of PEG-derivatized phospholipids, which reduce the uptake by MPS, thereof prolonging the circulation times and making available abundant time for these liposomes to leak from the circulation through the leaky endothelium. Unlike, conventional liposomes, PEG-liposomes do not show dose dependent blood clearance kinetics [209]. Vesicles containing PEG-conjugated lipids at various concentrations, molecular weights, or various sizes of PEG-containing vesicles were reported to have different circulation times [81, 84, 210-212]. These kind of liposomal systems are generally used in the ligand-mediated drug targeting [213]. This stealth principle has been used to develop the successful doxorubicin-loaded liposome product that is presently available in the market as Doxil (Janssen Biotech, Inc., Horsham, USA) or Caelyx (Schering-Plough Corporation, Kenilworth, USA) for the treatment of solid tumors.

Cryptosomes is a liposomal composition for targeted delivery of drugs. The composition comprises poloxamer molecules and liposomes encapsulating one or more delivery agents. Poloxamers are polyethylene oxide (PEO)-polypropylene oxide (PPO)-polyethylene oxide tri-block co-polymers of different molecular weights. The hydrophobic PPO group in the middle links the two hydrophilic PEO groups. The hydrophilic PEO groups of a poloxamer, on either side of the central PPO unit, can provide steric protection to a bilayer surface. The amphiphilic nature of the poloxamers makes them extremely useful in various applications as emulsifiers and stabilizers. It is considered that the central PPO unit, being hydrophobic, would tend to push into the bilayer interior serving as an anchor. Dislodging the poloxamer molecule from the bilayer is achieved by reducing its hydrophobicity which is achieved by decreasing the temperature. In an aqueous medium, poloxamers stay as individual molecules at temperatures below their critical micelle temperature (CMT), but at temperatures above the CMT, they form micelles due to their amphiphilic nature. In the presence of lipid bilayers, some poloxamer

molecules would partition into the bilayers as well as forming micelles with other poloxamer units. If the temperature again goes below the CMT, the poloxamer molecules lose their amphiphilic nature and disassociate from the lipid bilayer or micelle [179].

Emulsome, having the characteristics of both liposomes and emulsions, is a novel lipoidal vesicular system with an internal solid fat core surrounded by phospholipid bilayer. Emulsomes comprise a hydrophobic core (composed of solid fates instead of oils) as in standard oil-in-water emulsions, but the core is surrounded and stabilized by one or more envelopes of phospholipid bilayers as in liposomes allowing water insoluble drugs in the solution form without requiring any surface active agent or co-solvent. Emulsomes differ from liposomes since their internal core is a lipid, whereas the internal core in liposomes is an aqueous compartment. The drug loading is generally followed by sonication to produce emulsomes of smaller size [214]. These systems are often prepared by melt expression or emulsion solvent diffusive extraction. The lipid assembly of emulsomes, stabilized by cholesterol and soya lecithin (5-10% by weight), has features that are intermediate between liposomes and oil-in-water emulsions droplets. Emulsomes provide the advantages of improved hydrophobic drug loading in the internal solid lipid core and the ability of encapsulating water-soluble medications in the aqueous compartments of surrounding phospholipid layers.

Beside the other vesicular formulations, emulsomes are much stabilized and nano range vesicles. It is a new emerging delivery system and therefore could play a fundamental function in the effective treatment of life-threatening viral infections and fungal infections such as hepatitis, HIV, Epstein-Barr virus, leishmaniasis, etc. appear promising for the treatment of visceral leishmaniasis specifically and hepato-splenic candidiasis [214-216]. Emulsomes could be utilized in order to improve oral controlled delivery of drug, vaccine, and biomacromolecules. It is due to the fact that they are nano sized in range and could be utilized for the intravenous route. The common application areas of emulsomes are drug targeting, anti-neoplastic treatment, leishmaniasis (a disease in which a parasite of the genus *Leishmania* invades the cells of the liver and spleen) treatment, and biotechnology. Moreover, emulsomes could represent a more economical alternative to current commercial lipid formulations for the treatment of viral infections and fungal infections. Emulsomes provide a controlled and sustain release of drug. In comparison to the liposomes, emulsomes provide a prolong release of drug up to 24 hours, whereas liposomes have shown release up to 6 hours [217-219]. Emulsomes are nano size range in comparison to other vesicular delivery system such as niosomes and ethosomes. Due to the reduced size (10-250 nm) they can be used to enhance bioavailability to drug and as the best carrier for the intravenous drug delivery as well as oral drug delivery.

The lipid core of emulsomes may contain one or more anti-oxidants which are generally α -tocopherol or its derivatives that are the members of Vitamin-E family. The presence of anti-oxidants reduces the formation of oxidative degradation products of unsaturated lipids such as peroxides. The need of anti-oxidant can be prevented by the usage of saturated fatty acids during the preparation of the lipid core [220]. In the formation of emulsomes, like in the case of liposomes, cholesterol is essential component for the system that influences the stability of emulsomal systems and plays an important role in the drug encapsulation [221-224].

The most important advantage of emulsomes is their ability to protect the encapsulated drug from harsh gastric environment of stomach before oral administration because the drug is inside the triglyceride lipid core which can be supported that the gastric pH and the gastric enzymes are unable to hydrolyze triglycerides. Also, they resist development of multi drug resistance, often associated with over expression of a cell membrane glycoprotein, which cause efflux of the drug from the cytoplasm and results in an ineffective drug concentration inside the cellular compartment [225].

The development of emulsomes, however, is still largely empirical, and in vitro models that are predictive of oral bioavailability enhancement are lacking. There is a need for in vitro methods for predicting the dynamic changes involving the drug in the gut in order to monitor the solubilization state of the drug in vivo. Attention also needs to be paid to the interactions between lipid systems and the pharmacologically active substance. The characteristics of various lipid formulations also need to be understood, so that guidelines can be established that allow identification of suitable candidate formulations at an early stage. Future research should involve human bioavailability studies as well as more basic studies on the mechanisms of action of this fascinating and diverse group of formulations.

Unilamellar vesicles or liposomes are commonly used as simple cell models and as drug delivery vehicles to follow the release kinetics of lipophilic drugs that require compartmental models in its therapeutics and triggers. The localization of the drug at the site of action, rate of achieving the therapeutic index and circulation lifetime are the key parameters for a liposome. Lately, their arises a need for a multi-compartment structure consisting of drug-loaded liposomes encapsulated within another bilayer, is a promising drug carrier with better retention and stability due to prevention enzymes or proteins reaching the interior bilayers. A vesosome is a more or less heterogeneous, aggregated, large lipid bilayer enclosing multiple, smaller liposomes that offer a second barrier of protection for interior compartments and can also serve as the anchor for active targeting components [226, 76]. The multi-compartment structure of vesosome can also allow for independent optimization of the interior compartments and exterior bilayer; however, just the bilayer-within-a-bilayer structure of the vesosome is sufficient to increase drug retention from minutes to hours [227, 228].

In nature, eukaryotes increased their ability to optimize their response to their surroundings by developing multiple compartments, each of which has a distinct bilayer membrane, usually of quite varied composition and physical structure. Mimicking this natural progression to nested bilayer compartments led to the development of the vesosome, or vesicles deliberately trapped within another vesicle. The vesosome has distinct inner compartments separated from the external membrane; each compartment can encapsulate different materials and have different bilayer compositions. In addition, while it has proven difficult to encapsulate anything larger than molecular solutions within lipid bilayers by conventional vesicle self-assembly, the vesosome construction process lends itself to trapping colloidal particles and biological macromolecules relatively efficiently [229, 230]. The nested bilayer compartments of the vesosome provide a degree of freedom for optimization not possible with a single membrane enclosed compartment and a more realistic approximation of higher order biological organization.

The vesosome structure could be used to deliver a cocktail of antibiotics or antimicrobials to sites at a fixed ratio; such mixtures have been shown to act synergistically when delivered in a single liposome [231]. Such multi-drug formulations may be useful to avoid inducing pathogen resistance to a single drug.

As vesosomes are simply liposomes within liposomes, it should be possible to directly translate the extensive body of research on liposome drug delivery to the vesosome with only minor changes, and perhaps significant major improvements. The vesosome is created by simply self-assembly steps very similar to those used in making conventional unilamellar liposomes [229]. An important question is whether such additional effort in developing new structures will provide a therapeutic benefit over direct injection of the free drug or drug delivery by conventional unilamellar liposomes. The most obvious potential application for the vesosome is for drugs that have already shown increased efficacy by delivery with conventional liposomes. As an example, ciprofloxacin (cipro), a synthetic bactericidal fluoroquinolone antibiotic with broad spectrum efficacy, is released much more quickly from unilamellar liposomes in serum relative to saline [232, 233]. Conventional pH-loaded liposomes can retain essentially all encapsulated ciprofloxacin when stored in buffer for 12 weeks at 21 °C and 8 weeks at 37 °C [234, 235]. Although liposomal cipro has shown increased efficacy due to prolonged residence of cipro in the blood (free cipro is cleared in minutes), the half-life of release from the liposomes was only 1 hour, yet the liposomes themselves circulated for more than 24 hours [232, 235]. A second example is vincristine, a naturally occurring dimeric catharanthus alkaloid that has been used extensively as an antitumor agent since 1960's. The therapeutic activity of vincristine is dictated by the duration of therapeutic concentrations at the tumor site [236, 238, 239]. However, conventional liposomes, while offering improved bioavailability, also cannot encapsulate vincristine for sufficient time to give optimal results [234, 236, 237]. Future work will determine if multiple compartment structures like vesosome give sufficient enhancement of small drug entrapment to lead to new therapeutics.

Genetics play an increasingly important role in medicine and is used routinely to diagnose diseases and to understand malfunctions at the molecular level. The active approach of trying to amend genetic defects or insufficiencies is a logical next step. Major elements in the successful advance of gene therapy are identification of the disease and target cells, tissues and organs as well as construction of appropriate gene vectors, effective gene transfer and expression in the targeted cells. Many inherited diseases follow the Mendelian inheritance pattern in which the cause is due to a single genetic defect. Because the existing therapeutic treatments of such diseases are in most cases very limited, it is hoped that by transfecting appropriate cells with the correct gene or by adding a missing one, the disease could be alleviated. Examples of such potential treatments are for cystic fibrosis, hemophilia, sickle cell anemia or hypercholesterolemia and mutant tumor suppressor genes.

The aim of gene therapy is to deliver DNA, RNA or antisense sequences to appropriate cells in order to alleviate symptoms or prevent the occurrence of a particular disease, i.e. repair the defect and also its cause. The major approaches to gene therapy include gene replacement, addition of genes for production of natural toxins, stimulation of the immune system or over

expression of highly immunogenenic genes for immune self-attack and sensitization of cells to other treatments.

Recently, the studies on gene delivery into eukaryotic cells by the use of non-viral-lipid-based macromolecular delivery systems have been experiencing a growing interest owing to the appearance of clinical protocols for gene therapy. Although the efficiency and specificity of such non-viral delivery systems are not yet very high, some of the problems concerning transfection methods are being successfully solved. To date, the transfection mediators that ensure effective and directed gene delivery into various cells have been created. Transfection of plasmid DNA is closely connected to the problem of condensation of its molecule since the plasmid is too large (13-15 kb) to effectively overcome the cellular membrane barrier. Besides, free DNA has to be protected from destruction by endogenous nucleases. Lastly, it is necessary to neutralize the negative charge on DNA.

Genosomes are the artificial functional complexes for functional gene or DNA delivery to cell [238]. For the production of genosomes, cationic phospholipids were found to be more suitable because they possess high biodegradability and stability in the blood stream. Gene delivery is a vast area of research and a detailed summary of work in that field is beyond the scope of this chapter.

New generation liposomes and their features are summarized in Table 2.

Type	Main constituent	Advantage
Liposomes	Phospholipids	
Archaeosomes	One or more lipids containing diether linkages	High stability at several conditions
Niosomes	Non-ionic surfactant and cholesterol	Less prone to action of bile salts
Novasomes	Monoester of polyoxyethylene fatty acids, cholesterol and free fatty acids. Two to seven bilayer shells	High loading of drugs
Transfersomes	Lipid supramolecular aggregates	More flexible hence better transdermal delivery
Ethosomes	Phospholipids and alcohol in relatively high concentration	More disruptive in the skin lipid bilayer organization hence better transdermal delivery
Virosomes	Lipids surface modified with fusogenic viral envelope proteins	Intracellular delivery of antigens, drugs and DNA
Cryptosomes	Phospholipids and polaxamers or PEG	More stable
Emulsomes	Internal solid fat core surrounded by phospholipid bilayer	Better for encapsulation of hydrophobic drugs
Vesosomes	Multilamellar liposomes	Multidrug formulations are possible
Genosomes	Complex of cationic phospholipids and a functional gene or DNA	Suitable for gene delivery

Table 2. New generation liposomes and their features.

Extensively motivated by the need to increase the stability and bioavailability of drugs, and to reduce their side effects by targeting to the site of action, research in new drug delivery vehicles has taken giant steps. Liposomes and their derivatives, so called new generation liposomes, present a vast area in this field where several advances have already been achieved as summarized in this chapter. However, still further research is required to overcome the limitations faced today in terms of prolonged stability, drug loading and active targeting.

8. Conclusion

In the last decade from the concept of clinical utility of liposomes to their recognized position in mainstream of drug delivery systems, the path has been long and winding. The liposome systems have been explored in the clinic for applications as diverse as sites of infection and imaging, for vaccine, gene delivery and small molecular drugs, for treatment of infections and for cancer treatment, for lung disease and for skin conditions etc. Several liposomal formulations are already on the market, while quite a few are still in the pipeline for treatment of diseases. Conventional techniques for liposome preparation and size reduction remain popular as these are simple to implement and do not require sophisticated equipment. However, not all laboratory scale techniques are easy to scale-up for industrial liposome production. Many conventional methods, for preparing small and large unilamellar vesicles, involve use of either water miscible/immiscible organic solvents or detergent molecules. The need for improvements in the design and stability of liposomal diagnostic and therapeutic systems will continue to motivate innovative and efficient routes to their production.

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