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Review Article

LIPOSOMES: A NOVEL DRUG DELIVERY SYSTEM

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ABSTRACT

Almost from the time of their discovery the demonstration of their entrapment potential, liposomal vesicles have drawn attention of researchers as potential carriers of various bioactive molecules that could be used for therapeutic applications. Liposomes have been widely investigated since 1970 as drug carriers for improving the delivery of therapeutic agents to specific sites in the body. Liposomes, which are biodegradable and essentially non-toxic vehicles, can encapsulate both hydrophilic and hydrophobic materials, and are utilized as drug carriers in drug delivery systems. As a result, numerous improvements have been made, thus making this technology potentially useful for the treatment of certain diseases in the clinics. Many liposome-based DNA delivery systems have been described, including molecular components for targeting given cell surface receptors or for escaping from the lysosomal compartment. The insight gained from clinical use of liposome drug delivery systems can now be integrated to design liposomes that can be targeted on tissues, cells or intracellular compartments with or without expression of target recognition molecules on liposome membranes. The success of liposomes as drug carriers has been reflected in a number of liposome-based formulations, which are commercially available or are currently undergoing clinical trials.

Keywords : Liposomes, Gene Delivery, Phospholipid.

INTRODUCTION

The method by which a drug is delivered can have a significant effect on its efficacy. Some drugs have an optimum concentration range within which maximum benefit is derived, and concentrations above or below this range can be toxic or produce no therapeutic benefit at all. On the other hand, the very slow progress in the efficacy of the treatment of severe diseases, has suggested a growing need for a multidisciplinary approach to the delivery of therapeutics to targets in tissues. From this, new ideas on controlling the pharmacokinetics, pharmacodynamics, non-specific toxicity, immunogenicity, biorecognition, and efficacy of drugs were generated. These new strategies, often called drug delivery systems (DDS), are based on interdisciplinary approaches that combine polymer science, pharmaceutics, bioconjugate chemistry, and molecular biology. To minimize drug degradation and loss, to prevent harmful side-effects and to increase drug bioavailability and the fraction of the drug accumulated in the required zone, various drug delivery and drug targeting systems are currently under development. Among drug carriers one can name soluble polymers, microparticles made of insoluble or biodegradable natural and synthetic polymers, microcapsules, cells, cell ghosts, lipoproteins, liposomes, and micelles. The carriers can be made slowly degradable, stimuli-reactive (e.g., pH- or temperature-sensitive), and even targeted (e.g., by conjugating them with specific antibodies against certain characteristic components of the area of interest). Targeting is the ability to direct the drug-loaded system to the site of interest. Two major mechanisms can be distinguished for addressing the desired sites for drug release: (i) passive and (ii) active targeting. An example of passive targeting is the preferential accumulation of chemotherapeutic agents in solid tumors as a result of the enhanced vascular permeability of tumour tissues compared with healthy tissue. A strategy that could allow active targeting involves the surface functionalization of drug carriers with ligands that are selectively recognized by receptors on the surface of the cells of interest. Since ligand–receptor interactions can be highly selective, this could allow a more precise targeting of the site of interest. For over 20 years, researchers have appreciated the potential benefits of nanotechnology in providing vast improvements in drug delivery and drug targeting. Improving delivery techniques that minimize toxicity and improve efficacy offers great potential benefits to patients, and opens up new markets for pharmaceutical and drug delivery companies. Other approaches to drug delivery are focused on crossing particular physical barriers, such as the blood brain barrier, in order to better target the drug and improve its effectiveness; or on finding alternative and acceptable routes for the delivery of protein drugs other than via the gastro-intestinal tract, where degradation can occur.

Liposomes are a form of vesicles that consist either of many, few or just one phospholipid bilayers. The polar character of the liposomal core enables polar drug molecules to be encapsulated. Amphiphilic and lipophilic molecules are solubilized within the phospholipid bilayer according to their affinity towards the phospholipids. Participation of nonionic surfactants instead of phospholipids in the bilayer formation results in niosomes. Channel proteins can be incorporated without loss of their activity within the hydrophobic domain of vesicle membranes, acting as a size-selective filter, only allowing passive diffusion of small solutes such as ions, nutrients and antibiotics. Thus, drugs that are encapsulated in a nanocage-functionalized with channel proteins are effectively protected from premature degradation by proteolytic enzymes. The drug molecule, however, is able to diffuse through the channel, driven by the concentration difference between the interior and the exterior of the nanocage. Liposomes have the distinct advantages of being both nontoxic and biodegradable because they are composed of naturally occurring substances. Biologically active materials encapsulated within liposomes are protected to varying extent from immediate dilution or degradation, suggesting drug carrier systems for the transport of drugs and other bioactive capsules to disease-affected organs. The unique ability of liposomes to entrap drugs both in an aqueous and a lipid phase make such delivery systems attractive for hydrophilic and hydrophobic drugs. Because of advancements in the methods of preparing and formulating liposomes, high-entrapment efficiencies are possible for incorporating drugs into liposomes, creating a tremendous pharmaceutical impact. Furthermore, such encapsulation has been shown to reduce drug toxicity while retaining or improving the therapeutic efficacy. Several laboratories have reported the use of liposomes as drug carriers in the treatment of cancer, [1,2] leishmaniasis,[3] metabolic disorders, and fungal diseases.[4] Innovative research in liposomal drugs has led to commercialization of several anticancer therapeutics such as Doxil, Myocet, two liposome-based anticancer drugs; doxorubicin; and an antifungal drug formulation, AmBisome, which is a liposomal formulation of amphotericin B used for systemic therapy. Liposomes may have a use in gene delivery to correct gene-associated disorders or for vaccine therapy.[5] A quantitative entrapment of DNA can be achieved using the preparation of empty liposomes with cationic lipids followed by mixing with DNA or a plasmid of interest. Because of its convenience and efficacy, cationic lipid mediated gene delivery technology is a promising system for in vivo gene therapy. Clinical trials of large-size lipid–DNA complexes have mostly shown a lack of

adverse effects and moderate expression in a relatively low fraction of cells, but no decisive clinical disadvantages.

Classification of Liposomes

Liposomes can be classified either on the basis of their structural properties or on the basis of the preparation method used. These two classification system are in principle, independent of each other. The parameters for the first type of the classification are mention in the table 1. Dependent on the selection of lipids, the preparation technique, and preparation conditions, liposomes can vary widely in size, number, position of lamellae. These parameters influence the behaviour of liposomes both in vivo and in vitro. Classification based on method of liposome preparation is mentioned in the table 2.

Table 1 : Liposome classification based on structural features

MLV	Multilamellar large vesicles
OLV	Oligolamellar vesicles
UV	Unilamellar vesicles
SUV	Small unilamellar vesicles
MUV	Medium sized unilamellar vesicles
LUV	Large unilamellar vesicles
GUV	Giant unilamellar vesicles
MVV	Multivesicular vesicles

Table 2 : Liposome classification based on method of liposome preparation

REV	Single or oligolamellar vescile made by reverse phase evaporation method.
MLV / REV	Multilamellar vesicles made by reverse phase evaporation method.
SPLV	Stable plurilamellar vesicles.
FATMLV	Frozen and thawed MLV
VET	Vesicles prepared by extrusion method.
FUV	Vesicles prepared by fusion
FPV	Vesicles prepared by french press
DRV	Dehydration- rehydration vesicles
BSV	Bubblesomes

Mechanism of Liposome Formation

Liposomes are vesicular structures consisting of hydrated bilayers. Liposomes structures used for pharmaceutical purposes consist of a phospholipid baskbone. But other classes of molecules can form bilayer based vesicular structures as well. On the other hand not all the hydrated phospholipids form bilayer structures. Other forms of self aggregation such as inverted hexagonal phases or micelles with completely different properties can occur. The common feature that all bilayer forming compounds share is their amphilicity. They have defined polar and nonpolar regions. In water the hydrophobic regions tend to self aggregate and the polar regions tend to be in contact with the water phase. Israelachvili and coworkers defined critical packing parameter p by

$$P = v/ a_0 l_c$$

Where v is hte molecular volume of the hydrophobic part, a_0 is the optimum surface area per molecule at the hydrocarbon water interface, and lc is the critical half thickness for the hydrocarbon region which must be less tehn the maximum length of the extended lipid chains. For $p < 1/3$, spherical micelles are formed. In this category fall single chain lipids with large head group areas.eg lysophosphatidylcholine. For $1/3 < ½$ globular or cylindrical micelles are formed. Double chain "fluid state" lipids with large head area ($1/2 < p < 1$) form bilayers and vesicles. This occurs also with double chain "gel state" lipids with small head groups and $p \sim 1$. for $p > 1$ inverted structures such as the inverted hexagonal phase can be observed. An addtional condition required for bilayer formation is that the compound can be classified as a nonsoluble swelling ampiphile.

Raw Materials For Formation Of Liposomes

Liposomes that are used as carriers for drugs or diagnostic agents should be prepared from constituents that are safe for use in humans. Although limited experience is available on the safety of liposomes. Phosphatidylcholines and phosphatidylglycerols from natural sources, semisynthetically or fully synthetically produced and cholestrol and PEG-ylated phophatidyletanolamine, are frequently encountered in liposomes designed as drug carriers for parentral administration or for in vivo diagnostic purposes, Phosphatidylcholine (PC) is routinely used as a bulk neutral phospholipid . As a negatively charged lipid,phosphatidylglycerol (PC) is often selected. Finally , if it is desirable to reduce the permeability of "fluid crystalline state" bilayers, cholesterol is added to bilayer structure. Sometimes lipids with a special affinity for certain target cells in the body are deliberately inserted in bilayer. This was, for instance, the case when hepatocytic delivery was aimed for and lactosylceramide, a ligand with a special affinity for hepatocytes, was included in the liposomal bilayer. [6,7]

Five groups of phospholipids tthat can be used for the liposomal preparation can be discerned [8]

1. Phospholipids from natural soures
2. Modified natural phospholipids
3. Semisyntheyic phospholipids
4. Fully synthetic phospholipids
5. Phospholipids wiyh nonnatural head groups

Phospholipids from Natural Soures

The sources for natural phospholipids ,mainly PC, but also phosphatidylethanolamine(PE), phsphotidylinositol (PI),and sphingomyelin(SPM),are egg yolks and soyabeans. These PC's are mixed acyl ester phospholipids. Apart from source dependent diffrences in acyl chain type, considerable interbatch variation has been obsereved for egg PC.[9] The estrified acly chains of egg PC are diffrent from those of soybean PC.

Modified Natural Phospholipids

Natural phospholipids can be modified. Because of theor degreee of unsaturation, ehich makes them sensitive to oxidation, PC from natural sources ca nbe catalytically hydrogenated. Partially or fully hydrogenated natural PCs are readily available. The iodine value of these lipids is reduced as teh number of unsaturated C=C bonds drops. Dependent on the degree of unsaturation left after the hydrogenation preocess, phase transition temperatures canbe identified for liposomal dispersions of the patially hydrogenated PCs. Head group modofications can be performed by using phospholipase. With this enzyme one can convert PC into PG, PE or phosphatidylyserine (PS).

Semisyntheyic Phospholipids

These are acyl chains that are attached to phospholipds from natural sources are often unsaturated. This makes them liable to oxidation reactions, which may limit liposome shelf life. Moreover as mentioned above reproducibilty of the quality of the batches in terms of acyl chains may be poor as well, which may cause variation in stability or liposome properties. Removal of teh original acyl chain and within certasin limits, replacement by a chosen acyl chain is possible. Phospholipase A_2 which cuts the acyl chain at the C_2 position of glycerol can be used if only replacement of the C_2 acyl chain is required.

Fully Synthetic Phospholipids

Eibyl reviewed different completely chemical pathways for phospholipid synthesis[10].

Phospholipids with Nonnatural (Head) Groups

The idea of maintaining the fate of liposomes in the body by selecting the appropriate bilayer characteristics has led to modified phospholipids. The circulation time of liposomes in the blood compartment can be considerably prolonged when polyethleneglycol chains are attached to bilayer constituents. Alternatively, for active targetting purposes ligands for cell surface receptors canbe attached. These ligands can be chemically and

physically widely different structures, such as monoclonal antibodies or just a simple peptide. PEG has been linked to PE for the preparation of long circulating liposomes. Various reactions schemes have been developed. Molecular weights fractions for maximum prolongation of circulation times for PEG vary between 1900 and 5000. allen and cooworkers [11] described the synthesis of a PEG- carbonate derivative of PE. Klibanov et al. [12] used a succinidyl comjugation method, while blume and Cevc [13] adopted the procedure that Abuchowski and coworkers described for the preparation of PEG- albumin conjugates (via cyanuric chloride)

Techniques of Liposome Preparation

Liposome preparation techniques have been described extensively in a number of review articles. [8,9,14] In different preparation procedures a general pattern can be descerned 1. the lipid must be hydrated, then 2. liposomes have to be sized, and finally 3. nonencapsulated drug has to be removed. In some preparation schemes the hydration and sizing steps are combined. Sometimes all drugs are liposomes associated and no free drug can be found after stage2 then stage 3 is lacking.

1) **Hydration stage**

a) Mechanical Methods : MLVs were traditionally produced by hydrating thin lipids films deposited from an organic solution on a glass wall by shaking at temperatures above the phase transition temperature of the phospholipid with the highest T_c. The wide size distributions of the produced liposome dispersions were usually narrowed down by (low) pressure extrusion or ultrasoncation.[9].

b) Methods based on replacement of organic solvent by aqueous media : The lipid constituents are first dissolved in an organic solvent which is subsequetnly brought in contact with an aqueous phase. The organic solvent is removed later. During the removal of the organic phase, liposome are formed. Their characteristics (size, organisation of bilayers) depend on the protocol used. If the organiic solvent with the dissolved lipids is not miscible with the aqueous phase (ether, chloroform, freons) , then the intrmediate stage is an emulsion (immiscible solvent). Other organic solvents containing the dissolved lipid (s) can be mixed homogenously with the aqueous phase(ethanol) in the first stage. Then lipoosmes formation occurs when the organic solvent concentration drops below a certain critical value (miscible solvents). The contents of residual organic solvent that is exceptable in the finished product depends on the solvent in question and the route of administration. Apart from evaporation , techniques similar to those used to remove nonencapsulated material can be selected : gel permeation, ultracentrifugation, dialysis. Organic solvent may contain impurities wiht a high affinity for bilayers ; they may be enriched in the bilayer and cause safety or stability problems. Diethyl ether for instance, can be contaminated with peroxide that accumulates in the bilayer. Freshly (from bisulphite) distilled ether should therefore be used.

c) Methods based on detergent removal : (Phospho)lipids, lipophilic compounds and amphiphatic proteins can be solubilized by detergents forming mixed micells. Upon removal of the detergent, vesicle formation can occur. This technique is well established for preparation of reconstituted virus envelopes [21] or reconstituted tumor membrane material.[15] Schreier and coworkers described a two step strategy for insertion of proteins into the outer layer of liposomes. First liposomes were formed by detergent dialysis method and subsequently proteins were inserted by partial resolubalization of the membrane by the detergent (deoxycolate) in the presence of protein. [16]

d) Method based on size transformation and fusion : Sonication of phospholipds below their phase transition temperature (T_c) results in vesicles with defects in the bilayers. Heating the dispersion to T_c eliminates these structural defects and causes fusion resulting in large unilamellar liposomes with a wide size distribution.[17] Main disadvantage of this process is the limited number of bilayer composition that reacts and the poor reproducibility of the particle size distribution of the liposome dispersion that is formed.

2) **Sizing stage**

There are two approaches, one without a special sizing step [A] and one with a special sizing step [B]

[A] In liposome formation process, circumstances are selected and controlled in such a way that particle size distributions with an acceptable width are produced. High shear homogenization produces a size distribution which depends on operational pressure. [18, 19, 20]

[B] For small dispersion volumes, the liposome dispersion can be fractionated by centrifugation as liposome density usually differs from the density of the medium. Gel permeation chromatography has also been used for subdividing wide particle size distribution. On an analytical or semi-preparative scale, the selection of the pore size of the chromatographic material provides an opportunity to manipulate the size class resolution within certain limits.

3) **Removal of nonencapsulated material**

Many lipophilic drugs exhibit a high affinity to the bilayer and are completely liposome associated. However, for other compounds, the encapsulation efficiency is less than 100 percent. The nonencapsulated fraction of the active compound can cause unacceptable side effects or physical instability.[21] For removal of nonencapsulated material, the following techniques are used : a) dialysis and ultracentrifugation, b) Gel permeation chromatography, c) Ion exchange reactions.

Stability Of Liposomes

Liposomes face a number of chemical and physical destabilisation processes. So liposomes stability is an important consideration while studying liposomes. This aspect of liposomes stability have two aspects physical and chemical stability.

Physical stability

Physical processes that effect shelf life include loss of liposome associated drug and changes in size, aggregation and fusion. Aggregation is the formation of larger units of liposome material, these units are still composed of individual liposomes.In principle, this process is reversible ,e.g., by applying mild shear forces, or by changing the temperature or by binding metal ions that intiaaly induced aggregation. Fusion indicates that new colliodal structure were formed. As fusion is an irreversible process, the original liposomes can never be retrieved.

Drug molecules can leak from liposomes. The leakage rate strongly depends on the bilayer composition and the physiochemical nature of the drug. Bilayers in the gel state or those containig substantail (molar) fractions of cholesterol tend to lose assocaited drug only slowly; liquid state bilayers are more prone to drug loss and are less stable during storage. Bilayer permaebility is not necessarily a constant parameter. Chang in bilayer pernaebility can occur as a result of chemical degredation processes , such as the formation of lypo-PC and FA.

Chemical stability

a) Hydrolyiss of the ester bonds : Phosphatidylcholine possesses for ester bons. The two acyl ester bonds are most liable to hydrolysis. The glycerophosphate and phosphocholine ester bonds are more stable. The 1-acyl-lysophosphatidylcholine (LPC) and 2-acyl LPC are both formed at comparable rates.

b) Lipid peroxidatin of phospholipids: The polyunsaturated acyl chains of phospholipids are sensitive to oxidation via free radical reactions. Cyclic peroxides, hydroperoxides, malonilaldehyde, alkanes are the major degradation products. Low oxygen pressure, absence of heavy metals, addition of anti-oxidants, complexing agents (EDTA,etc), quenchers(beta-carotene) of the photo-oxidatio reactions improve resistance against lipid peroxidation.

Characterisation of Liposomes

Both physical and chemical characteristics of liposomes influence their behaviour in vivo and in vitro. [22] Liposome characterisation should be performed immediately after preparation. Different types of chromatography can be used to separate bilayer components (TLC,GLC,HPTLC). One major problem is detection and quantitation as UV molar absorptivity of lipids is low and depends on the degree of saturation of the acyl chains. For those phospholipids with only saturated acyl chains, alternative detection systems not based on UV absorption are described i.e. systems based on differential refractometry, light scattering and flame ionisation. The physical properties of liposomes have a direct impact on the behaviour of the liposomes with its content in vivo. Size, number of lamellae, internal mophology charge, bilayer fluidity are the factors that play a role in the in vivo disposition. Techniques for characterising size, number of lamellae, charge, and bilayer fluidity are listed in table 3.

Table 3 : Techniques for physical characterisation of liposomes

PARAMETER	TECHNIQUE
Size	Electron micrscopy light scattering ultracentrifugation Coulter counter
Namber Of Lamellae	NMR spectroscopy Small angle x-ray scattering Electron microscopy
Bilayer Fluidity	Fluorescence polarization
Charge	Microelectrophoresis

Liposome administered via the parentral route, on damaged skin, or in the eyes must be sterile. Heat sterelization by autoclaving, sterelization by γ-radiation, aseptic production procedures have been used to produce sterile products. Large liposome cannot be sterilized by heat therefore, should be manufactured aseptically. Assessment of the absence of pyrogens in loposome dispersions is difficult. It is not clear whether LAL(limulus amebocyte) test can be used for pyrogen detection.

Liposomes For Gene Delivery

It is important to dissect the overall cell uptake process into individual steps. In fact different studies have indicated that successful gene transfer in vitro involves: 1) the packaging of DNA, 2) the adhesion of packaged DNA to the cell surface, 3) internalization of DNA, 4) escape of DNA from endosomes if endocytosis is involved, 5) DNA expression in cell nuclei. To perform all of the above steps, liposomes have been explored as a delivery system for DNA as early as in 1979. [23] The encapsulation of plasmid DNA into liposomes [24] and the introduction of poliovirus RNA and SV40 DNA into cells via liposomes [25,26] were reported between 1979 and 1980.

Ph-Sensitive Liposome Strategy

Liposomes of various compositions can extensively bind to cell surfaces. For gene transfer, it was established that dioleylphosphatidylethanolamine (DOPE) is by far the most efficient lipid for in vitro gene transfection for pH-sensitive liposomes or as lipid helper in cationic liposomes. [27,28,29,30] It has been assumed that the function of phosphatidylethanolamine (PE) is that of a membrane fusion promoter, since in fact this lipid undergoes changes upon acidification. [31] Cholesterol is often essential to achieve sufficient stability of these liposomes. The composition of liposomes may play an important role in their interactions with cells. The size of liposomes and the type of cells are fundamental for an efficient capture by cells. Generally, liposomes are taken up by various endocytosic processes. Professional phagocytes such as macrophages and neutrophils can take up liposomes of various size and charge through active phagocytosis. The vesicular pathway for cellular uptake. After binding to the cell surface, liposomes are internalized into endosomes where they encounter a more acidic pH than in the external medium. Early endosomes generally have an internal pH of 6.50.[32,33] Their contents are then transferred to a more acidic environment by maturation or vesicular fusion. The last endosome environment, with an internal pH of 5.5-6.0, is reached 10- 15 min after uptake. The last endocytotic compartment, the lysosome, is further acidified to pH values of 5.0 or lower and is reached 20 min or more after uptake. The lysosomes are the main degrading compartment in the endocytotic pathway. Conventional, pH-insensitive liposomes and their content are delivered to lysosomes and degraded. The last requirement for plasmid liposomes after cell penetration is to avoid accumulation in particular cell compartments such as lysosomes. In order to prevent this degradation, pH-sensitive liposomes have been proposed.[27,28] pH-sensitive liposomes were designed based on the concept of viruses that fuse with the endosomal membrane by means of protein at pH 5-6, delivering their genetic material to the cytosol before reaching the lysosomes.[34,35] Generally, the lipid used to design pH sensitive liposomes is PE. PE represents a lass of lipids which, when dispersed in pure form, assemble into nonbilayer structures in n inverted hexagonal phase.[31] To stabilize PE in the lamellar hase in liposomes a series of stabilizers possessing titratable acid headgroup such as oleic acid (OA), palmitoylhomocysteine PHC) and cholesterolhemisuccinate CHEMS) were used. Liposomes composed of DOPE/OA/chol are capable of transfecting mouse Ltk-cells cells lacking thymidine kinase (TK)) with n exogenous TK gene. In this study, pHsensitive iposomes were 8-fold more efficient in gene delivery than pH-insensitive liposomes. Interestingly, the same investigators also demonstrated that plasmid DNA adsorbed to preformed empty pH-sensitive liposomes can transfect murine Ltk-cells in vitro. In contrast, negligible transfection by free plasmid DNA was observed. In a study by Zhou et al.,[36] pUCSV2 CAT DNA was used to prepare liposomes composed of DOPE/dioleylsuccinylglycerol (DOSG) (pH-sensitive formulation) or of dioleylphosphatidylcholine/DOSG (pH-insensitive formulation). The data showed that the acid sensitivity was directly related to the transfection activity. DOPE/DOSG liposome, which was the most sensitive to pH, transfected cells with the highest efficiency. Legendre and Szoka [37] compared the transfection efficiency mediated by pH-sensitive, pH-insensitive and cationic (DOPE/ dioleyloxypropyl-trimethylammonium bromide (DOTMA) liposomes using two different genes and five different cell lines. For all cell types investigated, cationic liposomes mediated the highest transfection level. While pH-sensitive liposomes mediated gene transfer, their efficiency was 1-30% of that obtained with DOPE/DOTMA and pH-insensitive liposomes did not induce transfection. It is important to emphasize the fact that separation of nonencapsulated (adsorbed or free) DNA was performed by pH-sensitive but not by cationic liposomes. This fact itself may interfere with the performance of the two types of liposomes. A key question remains concerning the mechanism of pH-sensitive liposomes: do they react as originally intended? Ropert et al. [38,39] encapsulated antisense oligonucleotides into pH-sensitive liposomes, a short length of DNA directed against the env gene of the murine Friend retrovirus, to inhibit virus proliferation. They suggested that the greater activity of oligonucleotides encapsulated into pH-sensitive liposomes was not due to a destabilization of the DOPE liposome bilayer but to an increased association between pH-sensitive liposomes and cells. They reported that the efficiency of the viral inhibition obtained with oligonucleotides encapsulated into pH-sensitive liposomes was only twice that of oligonucleotides encapsulated into non-pH-sensitive liposomes. And a two-fold increase in cell association was also observed when pH-sensitive liposomes were compared to pH-insensitive liposomes. In fact, pH-sensitive liposomes are taken up more efficiently by cells than pH insensitive liposomes, a fact probably leading to a better activity. [34]

Cationic Lipid Strategy

The encapsulation of DNA into conventional liposomes could be a technical problem due to the plasmid size, representing a poor transfection system. On this basis, an alternative technology based on cationic lipids and PE was developed in the late 1980s.[29] The idea was to neutralize the negative charge of plasmids with positively charged lipids to capture plasmids more efficiently and to deliver DNA into the cells. Generally, this is a simple procedure requiring mixing the cationic lipids with the DNA and adding them to the cells. This results in the formation of aggregates composed of DNA and cationic lipids. The cationic lipid DOTMA was first

synthesized and described by Felgner et al.[29] This lipid, either alone or in combination with other neutral lipids, spontaneously forms multilamellar vesicles (MLV) which may be sonicated to form small unilamellar vesicles (SUV). DNA interacts spontaneously with DOTMA to form DNA complexes with 100% of the DNA becoming associated. It is presumed that complex formation simply results from ionic interactions between the positively charged headgroup of DOTMA and the negatively charged phosphate groups of DNA. DOTMA is commercialized (Lipofectin., Gibco-BRL, Gaithersburg, MD) as a one to one mixture with DOPE and has been widely used to transfect a wide variety of cells.[40,41,42,43] In an effort to reduce the cytotoxicity of DOTMA, a series of metabolizable quaternary ammonium salts have been developed whose efficiency is comparable to that of Lipofectin when dispersed with DOPE.[44] As stated in the list of requirements, one important step for transfection is DNA compaction to improve cell penetration. Cationic amphiphiles able to compact genomic DNA, namely lipopolyamines, have been studied. Among them, DOSG (Transfectam.) has been shown to transfect many animal cells in a highly efficient manner.[45,46,47] These amphiphiles have been shown to stably condense DNA into particles. Common detergents of diverse structures (cetyl-trimethylammonium bromide (CTAB), dodecyl-trimethylammonium bromide (DDTAB)) have been compared for use in combination with DOPE. DDTAB seemed to be the most promising one and the DTAB/DOPE formulation was patented (TransfectACE.). As reported by Farhood et al.,[48] the role of DOPE in cationic liposome-mediated gene transfer seemed to be critical, and the compound has been extensively used. Since it has been postulated that the mechanism of DNA/cationic lipids uptake by cells is related to endocytosis, DOPE may favor the liberation of DNA into the cytosol as in pH-sensitive formulations. Electron microscopy observations have shown the endosome destabilizing effect of DOPE-containing cationic liposomes, although efforts to synthesize new cationic lipids led to the discovery of more efficient transfection agents, their efficiency does not correlate with their ability to deliver DNA after systemic administration to animals.[49] The physicochemical properties of the DNA/lipid complex may determine its stability in plasma and its biodistribution or pharmacokinetics. In an effort to determine the physicochemical properties of the complex, cationic lipids associated with DOPE and with various amounts of three different cationic surfactants have been investigated by cryo-transmission electron microscopy (TEM). [50] cyo-TEM analysis suggests that an excess of lipids in terms of charge leads to entrapment f the DNA molecules between the lamellae in clusters of aggregated multilamellar structures. The choice of surfactant does not appear to affect the morphology of the DNA-lipid-complexes. Furthermore, the system containing DOPE results in more compact aggregates than similar formulations using egg lecithin. Templeton et al. [48] have proposed a model for the assembly of DNA-lipid (N-1(2,3-dioleyloxy) propyl, N,N,N-trimethyl ammonium methyl sulfate- DOTAP)-chol complexes in which DNA adsorbs onto the invaginated and tubular liposomes via electrostatic interactions. This generates closed structures in which DNA may be protected. Farhood et al. [48] proposed the endocytosis s the major route for DNA-lipid complex uptake by cells during transfection. The surface-bound complex is internalized by endocytosis into endosomes and lysosomes n which a large part of the DNA would be degraded. According to Hui and Zhao,[52] the most evident pathway for DNA entry into CHO cells is also endocytosis and not direct fusion of the complex with the plasma membrane. Once inside the cell, how and then DNA and lipids become separate remains in question.

Liposome For Targeted Delivery

Use of liposome-encapsulated enzymes for delivery into cells was first reported in 1971. About the same time, a specific receptor on hepatocytes was demonstrated to mediate clearance of ß-galactose–terminated glycoproteins from circulation. A mannoside-specific receptor was recognized on the cell surface of the RES of rats (including the liver sinusiod and macrophages). By grafting different glycosides on the surface of liposomes, it is possible to direct the latter to different cell types of rat liver.[53] Galactosylated liposomes are mainly taken up by liver hepatocytes, whereas mannosylated liposomes are mainly taken up by nonparenchymal cells.[54] Grafting specific ligands to the liposome surface facilitates a fusion of the liposome with target cells by endocytosis, thus releasing material to be delivered. In cancer chemotherapy, the toxicity of anticancer drugs is of major concern. Liposomes could be used to deliver such drugs and minimize their toxic effects on healthy cells. Targeted delivery to cancer cells could be achieved by coating monoclonal antibodies (MAbs) raised against tumor-cell specific antigens. In vitro and in vivo studies by Ahmad et al. of squamous-cell carcinoma in mouse models provided evidence that antibody-coated polyethyleneglycol liposomes containing doxorubicin were more effective and less toxic than free drugs, drugs incorporated into antibody-free liposomes, and antibodycoated conventional liposomes [1,4]. The major concern in antibody-grafted liposome use is the induction of immune response to the grafted antibodies. Basten et al. suggested a novel approach to overcoming that difficulty.[55] They used 125I-labeled antigen to kill the cells responsible for immune induction (the “antigen suicide” technique). Other possible approaches to overcome the immune-system problem include immunosuppressive drugs and humanized antibodies or establishing neutral immune windows for subsequent injection. Liposomes can be designed to release their entrapped contents under certain controlled conditions: pH-sensitive and temperature-dependent liposomal systems.[1,56] Drug targeting using liposomes as carriers holds much promise, especially in reducing toxicity and targeting delivery to disease sites. The future is bright for liposome research, with a large number of clinical trials ongoing in several countries with liposomal formulations of various anticancer drugs, antisense, cytokines, peptides and proteins. In the near future, several more liposome-based drugs will find their way into the pharmaceutical market.

Liposomes In Cosmetics

In the past, the beauty enhancements expected from cosmetic products were obtained simply by combining the moisturizing, bleaching, or cell generating agents with the cosmetic base. Now cosmetic products have reached the stage where liposomes can encapsulate active ingredients thought to be necessary for the skin so they may be directly applied to the skin cells. Most useful for being able to transfer and deliver active ingredients to the application site of cosmetics. The liposome wall is very similar, physiologically, to the material of cell membranes. When cosmetic containing liposomes is applied to the skin, for example, the liposomes are deposited on the skin and begin to merge with the cellular membranes. In the process, the liposomes release their payload of active materials into the cells. As a consequence, not only is delivery of the actives very specific directly into the intended cells but the delivery takes place over a longer period of time. Liposomes are typically manufactured from various fatty substances that are used to encapsulate, or to create a sphere around, cosmetic materials. They act as a delivery system. Today, most of the experts working in the field of liposomal dispersions agree that liposomes do not penetrate as intact vesicles into the skin or permeate through the skin. Liposomes are believed to be deformed and transformed into fragments as a rule. Therefore size, shape, and lamallarity are not so relevant for the application, but for the chemical composition of the total formulation. The multifunctional properties of phosphatidylcholines lead to a number of different applications. So, formulations with unsaturated phosphatidylcholine are preferred to support skin regeneration, antiaging, acne preventing, and penetrating other active agents like vitamins and their derivatives into the skin. Formulations with hydrogenated phosphatidylcholine may be used for skin and sun protection, but it should be emphasized that in this respect nanoparticles and DMS are still more convenient. [57]

The numerous patents on liposome applications reflect the avid interest of many companies in this area. A patent involves a skin whitening lotion in which liposomes, consisting of vitamin E and complex lipids are dispersed in alcohol and water. The patent claims that vitamin E remains stable long enough to exhibit reducing action. A second patent related to skin whitening cosmetics involves the use of liposomes encapsulating ascorbic acid and alpha-tocopherol (vitamin E). This patent claims that the use of liposomes prevents the oxidation of ascorbic acid. The study showed that decomposition

of ascorbic acid was inhibited by the use of liposomes. One patent claims that the stability of a liposome is enhanced by covering its surface with fatty acid esters and polysaccharides. The next patent concerns a process for manufacturing large quantities of liposomes. Dissolving an amphiphilic substance in a solvent and spray drying the resulting solution gives rise to a fine powdery mixture. This powder, when dispersed in an aqueous medium and mixed, easily yields large quantities of liposomes. Phosphatidylserine, sphingomyelin or soybean lecithin may be used as the amphiphilic components, and yeast, antibiotics, elastin polypeptides, aloe vera etc., may be used as encapsulated active ingredients. Another patent claims that a membrane is formed by dissolving lecithin or phosphatidylcholine in an organic solvent which is then evaporated. The homogenization of this membrane with active ingredients can yield liposomes containing active ingredients which can be used in the manufacture of cosmetics. The patent also confirms the percutaneous absorption of the liposome's active ingredients through the use of a [sup.14] C label. Liposomal dispersions have proved not only to be innovative and effective cosmetic ingredients, but also to be a very convenient form to work with phosphatidylcholine. In dermatology, they will be used with success for preventing and treating several skin diseases. Complementary formulations are established where liposomal dispersions come up against limiting factors. Generally, members of the membrane family like liposomes, nanoparticles, and DMS are more compatible with the skin structure than usually applied conventional emulsions. "Compatible" means that formulations do not disturb the integrity of the skin lipid bilayers and are not washed out when the skin is cleaned. In the sense of modern strategies of cosmetics, these formulations get by with a minimum of auxiliary compounds, which put only a strain on the skin. Moreover, compatibility means embedding lipids and hydrophilic agents in the horny layer and being in line with the natural situation. Remarkably, phosphatidylcholine need not be applied in high concentrations, because experience shows that formulations are stable at lower amounts. Also, there is a cumulative effect in the horny layer with repeated application of phosphatidylcholine. In many cases liposomes, nanoparticles and DMS are compatible with each other in a sense that they can be used as a modular system. So these formulations are believed to still have a great future in cosmetic science. How far new findings about the importance of the choline moiety of phosphatidylcholine [58] will impact skincare research and development cannot be estimated.

Therapeutic Applications

1. Occular Application

The eye is protected by three highly efficient mechanisms (a) an epithelial layer that is a formidable barrier to penetration (b) tear flow (c) the blinking reflex. All three mechanisms are responsible for poor drug penetration into the deeper layers of the coenea and the aqueous humor and for the rapid wash out of drugs from the corneal surface. Enhanced efficacy of liposomes encapsulated idoxuridine in herpes simplex infected corneal lesions in rabbits was first reported in 1981.[59] Lee in 1985 concluded that ocular delivery of drugs could be either promoted or impeded by the use of liposome carriers, depending on the physiochemical properties of the drugs and lipid mixture employed. Ganglioside-containing liposomes and wheat germ agglutinin, a lectin that has a high binding affinity for both cornea and ganglioside, were tested for corneal adhesion.[60] Corneal binding as well as accumulation and transcorneal flux of carbachol was enhanced 2.5 to 3 fold over 90 min exposure times. Davies et al. [61] proposed the use of mucoadhesive polymers, carbopol 934P and carbopol 1342 to retain liposomes at the cornea. While precorneal retention times were indeed significantly enhanced under appropriate conditions liposomes even in the presence of the mucoadhesive had migrated toward the conjuctival sac with very little activity remaining at the corneal surface.

2. Pulmonary Application

Pulmonary delivery of liposomes has been explored as a target selective alternative to systemic administration of antiasthamatic and antiallergic compounds and for antibiotics used against pulmonary infections. Liposomes are useful tools for pulmonary delivery of drugs due to their solubilization capacity for poorly water soluble substances rendering them more practical for aerolisation. Their biodegradability allows for prolonged pulmonary residence times without danger of allergic or other side effects. The targeting capacity to infected or immunologically impaired alveolar macrophages is a unique feature of liposomes. The toxicity of liposomes aerosols has been investigated systematically. Gonzalez – Rothi et al. [62] found no inhibition of phagocytic activity or viability upon prolonged exposure of alveolar macrophages to liposomes. Padmanabhan et al [63] demonstrated high enzyme activity and prolonged tissue protection following pulmonary instillation of liposome in corporated superoxide dismutase and catalase although the mechanism respomsible for the observed protection remained obscure. Another promising application is the pulmonary application of liposome incorporated antimycobacterial drugs directed against Mycobacterium avium intracellulare (MAI) infected alveolar macrophages. Wichert et al [64] demonstrated the superior killing efficacy of liposomal amikacin against MAI in alveolar macrophage in vitro.

Clinical Applications

1. Cancer Therapy

Cytotoxic drugs can distribute non-specifically throughout the body, lead to death of normal as well as malignant cells, thereby giving rise to a variety of toxic side effects. Entrapment of these drugs into liposomes resulted in increased circulation lifetime, enhanced deposition in the infected tissues, 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 accumulation of liposomes in certain tumors in a higher concentration than found in normal tissues.[65,66] Many research efforts have been directed towards improving the safety profile of the anthracycline cytotoxics, doxorubicin (DXR) and daunorubicin (DNR), along with vincristine (VCR), which are associated with severe cardiotoxic side effects, although acute gastrointestinal effects and other toxicities may also occur. Liposomal entrapment of these drugs showed reduced cardiotoxicity, dermal toxicity and better survival of ex-perimental animals compared to the controls receiving free drugs.[65] Such beneficial effects of liposomal anthracyclines have been observed with a variety of liposomal formulations regardless of their lipid composition provided that lipids used high cholesterol (Cho) concentration or phospholipids with high phase transition temperature (Tc) are conducive to drug retention by the vesicles in the systemic circulation.[67] DXR entrapped in liposomes shows reduced non-specific toxicity and maintains or enhances anticancer effect. DXR hydrochloride constitutes the first liposomal product (DoxilTM) to be licensed in the United States. Surface grafted methoxypolyethylene glycol (MPEG) provides the hydrophilic stealth coating, which allows the DoxilTM liposomes to circulate in the blood stream for prolonged periods. The lipid matrix and an internal buffer system combine to keep virtually all the DXR encapsulated during liposome residence in the circulation. This means that the drug is not free to exert its toxic effects.[68] Liposome association alters the drug pharmacokinetics and thus the liposome has a half-life of approximately 55 hours in humans, whereas the free drug distributes to the tissues within a few minutes and is entirely cleared from circulation within 24 hours.[69] Liposomal formulation showed decreased toxic effects of DXR; a dose higher than the LD50 could be administered without acute toxicity, which suggests that these liposomes extravasate from the endothelium of tumor tissues and reside around tumor cells where they release the drug into the interstitial fluid. Preclinical and clinical investigations have demonstrated significantly increased efficacy and decreased toxicity of liposomes containing DNR (DaunoXomeTM) in comparison with free DNR [70] in the treatment of acute leukemia.[71] However, in the treatment of hepatocellular carcinoma and liver cirrhosis liposomal DNR showed mild

haematological toxicities and significant hepatic toxicity, which warns against further assessment of these liposomes in patients with hepatocellular carcinoma and liver cirrhosis.[72] However, liposomal DNR showed encouraging results in the treatment of advanced cutaneous T-cell lymphoma.[73] Furthermore, liposomal DNR and carboplatin plus etoposide, used to treat children with recurrent high-grade glioma after surgery and with progressive teratoid/rhabdoid tumor, showed encouraging results with only little and transient hematological toxicity.[74] Liposomal encapsulation of VCR resulted in increased and prolonged plasma concentration, which is associated with increased antitumor activity (murine P388 ascitic tumor) but not increased drug toxicities compared to the unencapsulated drug.[75] Guthlein et al. [76] found that VCR entrapped into a vesicular phospholipids gel consisting of densely packed liposomes was an effective delivery system with superior antitumor activity compared to conventional VCR against human small cell lung carcinoma LXFS 650 and the human mammary carcinoma MX1. Sustained release and passive tumor targeting can explain the enhanced efficacy.

2. Antimicrobial Therapy

Incorporation of rifabutin in liposomes resulted in a significant enhancement of activity against Mycobacterium avium infection compared to free rifabutin.[77] Moreover, the antitubercular activity of rifampin was considerably increased when encapsulated in egg phosphatidylcholine liposomes. A further increase in the activity was observed when the macrophage activator tetrapeptide tuftsin was grafted on the surface of drug-loaded liposomes. Rifampin delivered twice weekly for two weeks in tuftsin-bearing liposomes was at least 2,000 times more effective than the free drug in lowering the load of lung bacilli in infected animals.[78] Liposome encapsulated clarithromycin may be more effective than the free form against Mycobacterium avium intracellular (MAI) infections in vivo, and the use of a combination therapy with ethambutol could further enhance the efficacy.[79] Furthermore, when the activity of TLC G-65 (liposomal gentamicin preparation), alone and in combination with rifapentine, clarithromycin, clofazimine and ethambutol, was evaluated in the beige mouse model of disseminated Mycobacterium avium infection showed that the combination of rifapentine and TLC G-65 was more active than either agent alone. The activity of clarithromycin in combination with TLC G-65 was similar to that of either agent alone. Clofazimine improved the activity of TLC G-65 with respect to the spleen, while ethambutol improved the activity with respect to the liver.[80] Entrapment of ciprofloxacin in liposomes increases the circulation half-life of the drug when given by intravenous route in mice, which is associated with enhanced delivery of the drug to the liver, spleen, kidneys, and lungs. Furthermore, liposomal entrapment was associated with increased therapeutic efficacy against the Salmonella typhimurium infection model in mice.[81] Stevenson and coworkers [82] showed enhanced activity of streptomycin and chloramphenicol against Escherichia coli in the cells of the J774 murine macrophage line mediated by liposome delivery.

CONCLUSION

Liposomes have developed into a viable pharmaceutical dosage form. Progress has taken place in quantum leaps, rather than in a continuum, over the last two decades. Vital progess have been made in the development of long circulating liposomes that are not immediately recognized and removed by the cells of mononuclear phagocyte system. Despit this long circulating liposomes have opned a new realm of therapeutic opportuities and we will see a multitude of novel applications emerge in future. Development will continue to explore the validity of liposomes for the delivery of peptide and proteins, although progress in thid particular field has been meager. These developments will hopefully safeguard against the overoptimistic and unrealistic ideas and promises of the past and lead into another highly productive and innovative phase of liposome research.

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