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Liposomes as Drug Delivery Systems

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ABSTRACT

Liposomes were investigated for a long time as nanocarriers to target drugs to their sites of actions and to reduce toxicity. The unique structure of liposomes which is similar to the cellular phospholipids is considered as being an advantage to such delivery systems. Both hydrophilic and hydrophobic drugs can be encapsulated into the liposomes for delivery of a wide range of drugs such as anticancer drugs and antiinfective drugs used to treat tuberculosis and hepatitis. Liposomes can also be considered to delivery nutritional supplements such as vitamin C and vitamin D. Types of liposomes, lyophilization, advantages, disadvantages, challenges and immungenicity have been discussed.

Keywords: Liposomes; Anticancer; Anti-Infective; Nutritional Supplements

Abbreviations: RES: Recticuloendothelial System; PEG: Polyethylene Glycol; MLV: Large Multilamellar Vesicles; OLV: Oligolamellar Vesicles, GUV: Giant Unilamellar Vesicles, LUV: Large Unilamellar Vesicles; SUV: Small Unilamellar Vesicles; TAAs: Tumor-Associated Antigen; APCs: Antigen Presenting Cells; CTLs: Cytotoxic T Lymphocytes; TERT: Telomerase Reverse Transcriptase

Introduction

Targeted drug delivery to improve cellular uptake of drugs and reduce toxicity has advanced recently. Liposomes have been investigated for almost 60 years as nanocarriers to target drugs to their site of action [1]. They have been explored as drug delivery systems due to their unique structure which is similar to the structure of cellular phospholipids and the fact that liposomes can be formulated in different forms. Both hydrophilic and hydrophobic drugs can be encapsulated inside the core of the liposomes for the delivery of various drugs such as anticancer drugs and antiinfective drugs used to treat tuberculosis and hepatitis [1-3]. Furthermore, the large aqueous center and biocompatible lipid exterior permits the delivery of macromolecules, such as DNA, proteins and imaging agents. Liposomes have improved therapies for a range of biomedical applications by stabilizing therapeutic compounds, overcoming obstacles to cellular and tissue uptake, and improving biodistribution of compounds to target sties *in vivo*. As a drug delivery system, liposomes offer several advantages including biocompatibility, capacity and biophysical properties that can be modified to control their biological characteristics. Liposomal formulations are characterized by properties such as particle size, charge, number of lamellae, lipid composition, and surface modifications with polymers and ligands that govern their stability *in vitro* and *in vivo.*

Encapsulation within liposomes protects compounds from early inactivation, degradation, and dilution within the circulation. Liposomes are generally considered to be pharmacologically inactive with minimal toxicity, as they tend to be composed of natural phosolipids, however increasing number of studies have shown that liposomes are not as immunogenically inert as once suggested. Despite the success of liposomal formulations *in vivo*, their translation into the clinic has not progressed as expected [1].

Types of Liposomal Drug Delivery

There are four key types of liposomal delivery systems: conventional liposomes, sterically stabilized liposomes, ligand-targeted liposomes, and a combination of the above in terms of their composition (Figure 1). Conventional liposomes were the first type to be developed. They consist of a lipid bilayer that can be composed of cationic, anionic, or neutral phospholipids and cholesterol, which encloses an aqueous volume. The research on conventional liposomes to encap-

sulate drugs began in 1980s. Conventional liposomes reduced the toxicity of compounds *in vivo*, through modifying pharmacokinetics and biodistribution to enhance drug delivery to diseased tissue in the body. However, the delivery system was prone to rapid degradation from the bloodstream, therefore limiting its therapeutic efficacy. This rapid clearance was due to opsonization of plasma components and uptake by macrophages of the recticuloendothelial system (RES), mainly in the liver and spleen [1]. In terms of composition and mechanism of intracellular delivery into five types:

1. Conventional liposomes,
2. pH-sensitive liposomes,
3. Cationic liposomes,
4. Immunoliposomes, and
5. Long-circulating liposomes [4].

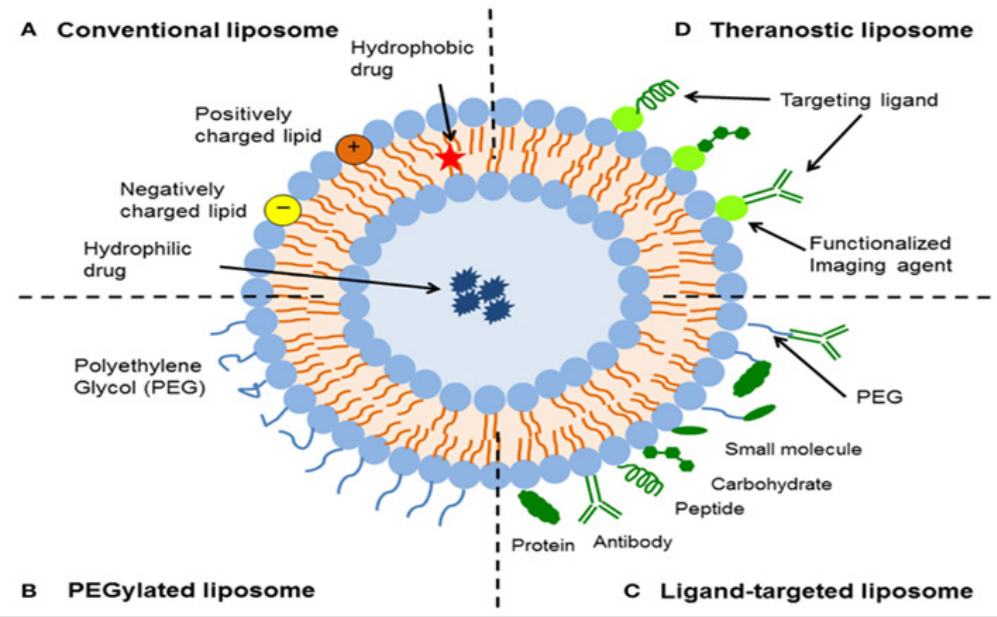

Figure 1: Schematic representation of the different types of liposomal drug delivery systems
A. Conventional liposomes – liposomes consisting of a lipid bilayer enclosing an aqueous core,
B. PEGylated liposomes – PEG is added to the surface of the liposomes to stabilize the liposome,
C. Ligand-targeted liposomes, ligands such as antibodies, peptidies, and carbohydrates are added to the liposome surface for specific drug targeting and
D. Therasonstic liposomes – a single system consisting of a nanoparticle, a targeting element, an imaging component, and a therapeutic component (Sercombe L, et al. [1]).

To improve liposomal stability and their circulation times in the blood, sterically stabilized liposomes were introduced. The hydrophilic polymer, polyethylene glycol (PEG), has been shown to be the optimal choice for obtaining sterically stabilized liposomes. The establishment of a steric barrier improves the efficacy of encapsulated agents by reducing *in vivo* opsonization with serum components and the rapid recognition and uptake by the RES. This not only reduces the elimination of the drugs by prolongation of blood circulation and providing accumulation at pathological sites, but also attenuates side effects. Steric stabilization strongly influences the pharmaockinetics of liposomes, with reported half-lives varying from 2 hours to 24 hours in rodents (mice and rats) and as high as 45 hours in humans, depending on the particle size and the characteristics of the coating polymer. While coating liposomes with PEG results in prolonged circulation times, there can be a reduction in the ability to interact with the intended targets [1]. Liposomes are good drug delivery systems for site-specific delivery of drugs to designated cell types or organs inside the human body. Many types of ligands such as antibodies, peptide/proteins and carbohydrates can be attached to the liposomes for such site-specific targeting. The coupling of antibodies, particularly monoclonal antibodies, to create immunoliposomes represents one of the more versatile ligands that can be affixed to liposomes surfaces.

One of the advantages of using monoclonal antibodies is their stability and higher binding avidity because of the presence of two binding sites on the molecule. The major disadvantages of such immunoliposomes are their poor pharmacokinetics and immunogenicity. In order to overcome such disadvantages, other types of immunoli-

posomes have been investigated. Attaching PEG to immunoliposomes have been found to be useful in overcoming such hurdles. Liposomes offer a good alternative to target drugs in various diseases [1]. The liposomes can be classified according to their size. The liposome size vary from 0.025 μm which is very small to 2.5 μm which is considered as large. Liposomes can also have one or bilayer membranes. The vesicle size affects the circulation half-life of liposomes, and both the size and number of bilayers affect the amount of drug that can be encapsulated into the liposomes. Liposomes can be classified according to their size and number of bilayers as follows:

1. Large multilamellar vesicles (MLV) with a size larger than 500nm,
2. Oligolamellar vesicles (OLV) with a size between 100 and 500nm
3. Unilamellar vesicles which can be further subdivided into giant unilamellar vesicles (GUV) with a size between 20 and 100nm, large unilamellar vesicles (LUV) with a size larger than 1000nm and small unilamellar vesicles (SUV) with a size larger than 100 nm [4-6].

Advantages and Disadvantages of Liposomes

Advantages of liposomes include delivery of hydrophobic, amphipathic and hydrophilic drugs, increased efficacy and therapeutic index of drugs such as actinomycin D, increased stability from the external environment through drug encapsulation, reduced drug toxicity e.g. amphotericin B and taxol. Liposomes are considered as non-toxic, biocompatible, completely biodegradable, and non-immunogenic for both systemic and nonsystemic administrations. They also help to reduce the exposure of sensitive tissues to toxic drugs. Target targeting is achieved by the flexibility of the liposomes to couple with site-specific ligands. The benefits of drug loading in liposomes include: improved solubility of lipophilic and amphiphilic drugs, passive targeting of the drugs to the cells of the immune system especially the mononuclear phagocytic system, sustained release of systemically or locally administered liposomes, site-avoidance mechanism, site-specific targeting, improved transfer of hydrophilic, charged molecules, and improved penetration into the tissues [4,6]. Disadvantages of liposomes include: allergic reactions may occur to liposomal constituents, short half-life, oxidation and hydrolysis-like reactions of the phospholipid bilayer, leakage and fusion of the encapsulated drug, production cost is high, and at some instances, and low solubility [4,6].

Lyophilization of Liposomal Formulations

In order to overcome liposomal limitations such as oxidation and/or hydrolysis of the phospholipids, drug leakage, formation of aggregates or vesicle fusion with consequent change of the biodistribution *in vivo* which affect safety and efficacy, lyophilization of liposomal formulations may be necessary. Typically during the process of lyophilization, the formulation undergoes freezing, primary drying, and secondary drying. The freezing phase occurs through cooling in order to separate the liposomes and excipients from the solvent resulting in the formation of ice crystals. Annealing of the frozen sample is introduced in order to decrease drying rate and sample heterogeneity. The primary drying is intended for solvent sublimation through increasing the temperature and decreasing the pressure. The secondary drying is the result of increasing the temperature in order to allow the water desorption. During the freezing and drying steps, stresses on the liposomal structure and drug encapsulation result in drug leakage and liposomal structural changes. In order to reduce such stresses, protectants are included in the outer aqueous phase of liposomal dispersions to stabilize the liposomal membranes.

Sugars, particularly disaccharides, are used along with liposomal dispersions for protection purposes [7]. The ability of the freeze-dried liposomal formulation to readily reconstitute upon the addition of appropriate solvent depends on several factors such as undesired physical structure of the freeze-dried powder leading to poor wetting, agglomeration, or prolonged reconstitution time. The morphology and size of the liposome should not change upon reconstitution. There are methods to monitor the morphology and size of liposomes once reconstitution takes place. These methods should be specified in the guidelines for producing lyophilized liposomes [7].

Anticancer Drug Delivery

Liposomes are important drug delivery systems that show a great potential in cancer treatment. The use of liposomes offers many advantages including biocompatibility, lower clearance rates, the ability to target cancer tissues, and controlled release of drugs. The clinical use of liposomes in cancer include delivery of drugs and diagnostic agents. Approval of the use of Doxil is a proof that liposomes are attracting much attention in the delivery of various drugs to tumor cells [3]. Liposomal drug delivery to tumor cells provide enhanced efficacy for anticancer agents due to enhanced permeability and retention effect as well as reduced toxicity. Liposomal anthracyclines such as liposomal daunorubicin and PEGylated liposomal doxorubicin have achieved highly efficient drug encapsulation, resulting in significant anticancer activity with reduced cardiotoxicity. PEGylated liposomal doxorubicin formulations have shown substantial efficacy in breast cancer treatment both alone and in combination with other drugs. The next generation of drug delivery systems include immunoliposomes and other ligand-directed constructs capable of tumor recognition [2]. Currently approved liposomal drug delivery systems provide a stable formulation, improved pharmacokinetics, and a degree of 'passive' or 'physiological' targeting to tumor tissues. Liposomes can also interact with plasma proteins and other cell membranes.

To target liposomes specifically to cancer cells, antibody-mediated or other ligand-mediated compounds are added to the surface of the liposomes. Immunoliposomes are liposomes conjugated to mAb fragements. The advantages of using immunoliposomes compared to

other antibody-based strategies include reduced side effects as well as targeting the drugs to cancer cell, longer circulation time even with repeated administration, and higher drug loading compared to immunoconjugate [2].

Use of Vaccines in Cancer Treatment

Tumor-associated antigen (TAAs)-specific T cells are detected in many patients with cancer. However, these TAA-specific T cells fail to control tumor growth. It is expected that vaccination with TAA enhances tumor-specific T cell responses, improves antitumor immunity, and provides clinical benefit. The main disadvantage preventing the availability of cancer vaccine is the immunogenicity of the antigen peptides used as vaccines. Liposomes are usually used as carriers of the antigens and are also adjuvants to induce immune responses. Liposomes containing unsaturated fatty acids can be crossed linked by antigen presenting cells (APCs) to cytotoxic T lymphocytes (CTLs). Surface-coupled liposomal antigens are still being examined for the development of cancer vaccines. Telomerase reverse transcriptase (TERT) is a tumor antigen that is highly expressed in more than 85% of all human types of cancer including stem cell-like tumor cells. As a result, TERT antigens are good candidates to mediate antitumor immune responses against a range of tumors. Future investigations should be conducted to address whether the telomerase inhibition by host immune system activated by immunotherapy can affect normal tissues [8].

Gene Delivery Systems in Cancer Treatment

Adenosine (Ad) is widely used for with anticancer agent as it has a few disadvantages such as premature neutralization by components of the immune system and inferior tropism towards cells. Thus, a primary objective in such drug delivery systems of adenosine is to overcome tissue tropism and overcome the immune system. In order to achieve this, formulators have to modify Ad vectors either physically or chemically with polymers such as polyethylene glycol (PEG), poly-N-(2-hydroxypropyl) methacrylamide (pHPMA) and poly(ethylenimine) (PEI). The shielding of Ad vectors with such polymers was observed to reduce liver toxicity and evade a variety of immune system responses *in vivo*. The main limitation of using such polymers is the reduced potential of adenosine to enter the target cells. To overcome such a limitation, a number of targeting moieties (e.g. tumor-homing peptides, growth factors, or antibodies) can be used to avoid unwanted transduction and enhance therapeutic efficacy. Alternatively, it has been shown that anionic liposomes as drug carriers of adenosine vectors are able to increase the transduction efficacy of adenosine and reduce cytotoxicity compared to cationic liposomes. However, liposomal drug delivery systems lack selectivity and specificity, and the liposomal systems can be inactivated in the presence of high titers of high anti-adenovirus antibody *in vitro*. Thus existing systems still need further modifications.

The use of PEG usually reduces the antigenicity and immunogenicity of proteins. However, PEGylation inhibits cellular uptake and affects intracellular trafficking resulting in significant loss of biological activity of the delivery system. For successful gene delivery in cancer treatment, the crucial issue of the use of PEG or the 'PEG dilemma' must be solved. Recently, matrix metalloproteases (MMPs) have attracted much attention owing to their ability to degrade the extracellular matrix (ECM), which is involved in the angiogenesis, invasion, and metastasis of malignant tumors. Type IV collagenases (MMP-2 and MMP-9) have been reported to play an important role in the mentioned process and the expression levels of MMPs were found to be relatively high in tumor cells compared to non-malignant tumors. An enzymatically cleavable PEG-lipid material composed of PEG/matrix metalloproteinase (MMP) – substrate peptide / cholesterol (PPC) is a way for protecting the liposome adenosine drug delivery system. The cleavable PEG can avoid the drawbacks of the traditional PEG modification and play a dual role for targeted delivery and immune protection. The influence of PEGylation on the vesicle size and size distribution is negligible, although the size of the vesicle slightly increased with increasing PEG grafting density.

The PEG chain could be shed from the liposomes when it reacts with MMPs. It was also noticed that envelopment by anionic liposomes and PEG modification of Ad reduces the ability of these vectors to induce Ad-specific neutralizing antibodies *in vivo* [9]. Successful immunotherapy against cancer requires the induction of a robust cytotoxic T lymophocyte response with the appropriate immune environment and T cell help. Antigens play a major role in the lymphocyte response. There are two different pathways for antigen cross presentation. In the cytosolic pathway, the internalized antigen reach the cytosol where they are degraded by the proteasome. Degradation products are then transported by antigen associated protein transporters to the endoplasmic reticulum or the lumen of endosomes or phagosomes thus forming histocompatible molecules. In the vacuolar pathway, internalized antigens are degraded by lysosomal proteases and then loaded into histocompatible molecules. Several studies have shown that protein loaded liposomal formulations activate T lymphocytes through the produced histocompatible molecules [10].

Delivery of Docetaxel and Palymitoyl Ascorbate for Synergistic Antitumor Efficacy

High dose ascorbate has been shown to reduce the incidence of most malignancies. High doses of orally administered ascorbate was shown to only marginally increase the plasma concentration of vitamin C. Thus, vitamin C should be given intravenously or through the encapsulation of ascorbate into a liposomal formulation. Due to the fact that ascorbate is not very stable in aqueous media, polmitoyl ascorbate is used as an ascorbate derivative. Palmitoyl ascorbate is not only more stable than ascorbate, it has been shown that palmitoyl ascorbate is more efficient in inhibiting the growth of tumor cells

[11]. increase their chemical stability and to deliver both drugs into the tumor cells. Co-administration of both poalmitoyl ascorbate and doxetaxel can be done by thin film hydration technique. The resultant liposomes are then nanosized (140 – 170nm). Nanosizing allows the palmitoyl ascorbate to efficiently accumulate into the tumor cells. The resultant liposomes also controlled the release of both ascorbate and doxetaxel. The combined use of palmitoyl ascorbate and doxetaxel at a weight ratio of 200:1 had the highest synergistic effect in HepG2, MCF-7 and PC-3 lines. The combination of both palmitoyl ascorbate and doxetaxel was shown to have a much better inhibition effect of the growth of cancer cells than either agent alone. Encapsulating both agents into a liposomal system provides a promising therapeutic strategy for tumor targeting and enhanced antitumor therapy [11].

The Use of Vitamin C-driven Epirubicin Liposomes in Cancer

Ascorbic acid (vitamin C) is a water-soluble vitamin with two ionizable groups. It has the ability to maintain iron in its ferrous state thus affecting the catalysis of various biochemical reactions due to the effect of ferrous iron. This oxidizing ability of vitamin C and its role in many physiological functions probably make vitamin C have a selective toxicity against cancer cells. Vitamin C can also enhance the antineoplastic activity of some anticancer drugs especially antracylcines such as epirubicin. It has been shown that intravenous vitamin C improves the lives of breast cancer patients during radiotherapy and chemotherapy. Vitamin C can also enhance the effect of doxorubicin in breast cancer patients over a wide range of vitamin C concentrations [12]. Liposomes are one of the leading intravenous drug delivery systems as shown for many drugs. Loading both vitamin C and epirubicin into the liposomes at specific molar concentrations specific for their antitumor activity will deliver the drug and vitamin C to the tumor cells. Peglylated liposomes were evaluated for the delivery of both vitamin C and epirubicin to cancer cells. Drug encapsulation was done via the ion/pH gradient method. These long circulating liposomes are known to accumulate inside the tumor cells through what is known as enhanced-permeability-and-retention (EPR) effect. Drug leakage from the liposomes to the tumor cells is affected by several factors such as liposome composition, drug-bilayer interaction, and encapsulation method.

If the external liposomal pH value is around 5.5, it was observed that the internal pH of the liposomes is 2.4, the release of liposomal contents into the cancer cells was better compared to when the internal pH of the liposomes is 4.0. The *in vivo* activity of the liposomal formulation was done in miche with 4T-1 marine mammary cancer, which closely resembles human breast cancer. Increased antitumor activity has been observed when epirubicin was encapsulated using an ammonium ascorbate gradient due to the increased circulation of the drug in the body. Both ascorbic acid and epirubicin have synergistic antitumor activity [12].

Infectious Diseases

When amphotericin was developed as a liposomal delivery system and used for the treatment of systemic fungal infection and leishmaniasis, it was found that amphotericin produced less nephrotoxicity so liposomal amphotericin could be given to patients with renal damage. Liposomal amphotericin was also found to be effective in treating systemic fungal infections in patients resistant to fluconazole and plain amphotericin. Even lower doses were needed when liposomal amphotericin was used compared to amphotericin alone for treating the same infection [2].

Liposomal Antigen Delivery Systems for Infectious Disease Treatment

Liposomes are possible vehicles for the delivery of antigens to the immune system. Some parameters, such as lipid composition and epitope density, influence the immunogenicity of haptenated liposomes and antigen-containing liposomes. As for the antigen-containing liposomes, there seems to be general principles concerning the immunogenicity of such liposomes, yet not all principles can be applied to every antigen-containing liposome. As the liposomal membrane fluidity decreases, the immunoglobulin G response increases. This effect disappears once a booster is given. The protein content did not have an effect on the immunogenicity [13]. Hepatitis C virus (HCV) causes persistent infections in more than half of infected patients which often lead to the development of cirrhosis and hepatocellular carcinoma. Pegylated interferon and ribavirin therapy, although beneficial in about half of treated patients, are expensive and associated with significant side effects. Antigens chemically coupled to the surface of liposomes consisting of unsaturated fatty acids were cross-presented by antigen presenting cells to cytotoxic T lymphocytes (CTIs). HCV-derived antigenic preparations peptides couples to the surface of liposomes serve as efficient vaccine vehicles for the induction of anti-viral immunity mediated by CD8 T cells [14]. Despite high coverage of Bacillus Calmette-Guerin (BCG) vaccination worldwide, the high burden of adult TB patients and Latent TB infection (LTBI) populations urgently requires the development of the next generation of TB vaccine.

Much progress has been made in this area during the recent decades and at least 50 vaccine candidates have been evaluated preclinically or clinically. However, there is still a long way to go to obtain a new licensed TB vaccine. Several antigens of M.Tuberculosis with low molecular weight have shown the ability to induce a CD8 T cell response. Liposomes are potential carriers of vaccines for tuberculosis treatment and need further preclinical and clinical evaluation [15].

Bile Salt-Coated Liposomes

Severe liver disease usually makes use of the hepatocycte pathology. In order to optimize treatment by targeting the drug into the liver without exposing other parts of the body to the drug to minimize tox-

icity, hepatotropic liposomes present a good choice. In order to direct the liposomes into the hepatocytes, a signal should be found on the surface of the liposomes. Glycoproteins like asialofetuin, Glycyrrhizin, and a soybean-derived sterylglucoside have been investigated to direct the liposomes into the liver. After targeting the liposomes to endocytosing receptors, internalization of the liposomes into liver lysosomes degrades the liposomes. Binding of lipsomes with bile salts like lithocholic acid will target the liposomes into the hepatocytes without causing their degradation by hepatic lysosomes [16]. The lipsomes can be prepared by the extrusion method. A bile salt-lipid conjugate was synthesized so as to be attached into the surface of the liposomes in an attempt to direct the liposomes into the hepatocytes. A phospholipid is the main binding block of the bile salt to the surface of the liposomes. Lithocholic acid was chosen as the bile salt as it has only one hydroxyl group that will allow further chemical modification of the liposomes without affecting other functional groups. Other bile salts with two or three hydroxyl groups such as cholic acid or ursodeoxycholic acid can also be used. They are less toxic than lithcholic acid but are more complex to synthesize.

Taurine-conjugated bile salts were also investigated as they have a negative charge that will prevent liposomal non-specific binding to the cells by preventing hydrophobic bile salts from being inserted into the surface of the liposomes at physiological pH values. The stereochemistry of the attached bile salt to the surface of the liposomes plays a role in further necessary modifications. The attached bile salts are also able to bind covalently to the surface of the hepatocytes in order to anchor the liposomes inside the liver. The mentioned interaction also depends on the anionic charge on the lipids found on the surface of the liposomes as well as the net charge of the liposomes. Thus the mentioned liposomes can be used for diagnostic purposes but for drug targeting purposes the membrane interaction should be overcome. One approach to overcome such interaction is the use of lipophilic prodrugs that can enter the hepatic cells through diffusion [16].

Proteoliposomes

Membrane proteins are important in many biophysical processes in the body. Almost one third of human gene code is for membrane proteins. Membrane proteins play a role in many diseases, so they are the subject of many current research. Reconstitution of such proteins into lipid bilayer vesicles is also the subject of much research. Reconstitution is affected by the protein, type of lipid, and the detergent used. The influenza M2 proteins are membrane bound and play an important role in uncoating of virons when the viruses enter the cell. There are two types of reconstitution for preparing M2 proteoliposomes which are organic co-solubilization of both the lipids and M2 and detergent-mediated reconstitution [17]. The direct insertion of influenza M2 proteins onto the liposomal surface involves four steps [17]:

1. Preparation of the liposomes with a defined size by extrusion
2. Saturation of the liposomes with detergent
3. The addition of protein-detergent micelles to the detergent-saturated liposomes
4. Removing the detergent
5. Detergent removal by six 15 minutes incubation with biobeads was sufficient to remove the detergent without the need for overnight incubation. Reconsitution was also found to be successful after efficient detergent removal [17].

Liposomes containing Polyethylene Glycol

Polyethylene glycol (PEG) is considered non-toxic and non-immunogenic. The attachment of PEG to proteins and nanocarriers, so-called PEGylation, lowers the ability of the immune system to identify such drug carriers. Despite such an argument, several disadvantages exist. PEGylated liposomes loose their prolonged circulating properties when injected repeatedly in the same animal. This is known as the accelerated blood clearance (ABC) phenomenon by which the first dose of PEGylated liposomes primes splenic B cells to mount an antibody response manifested by the production of anti-PEG IgM. The secreted anti-PEG IgM then binds to the PEG in a second dose of PEGylated liposomes and causes the rapid clearance of such liposomes via complement activation and enhanced uptake of the liposomes by the Kupffer cells. In addition, anti-PEG antibody production and rapid clearance of subsequent doses have been also reported upon various PEGylated materials such as PEGylated proteins, micelles, and PEGylated emulsions. The insertion of ganglioside into PEGylated liposomes substantially attenuates the anti-PEG IgM immune response and subsequently alleviates the incidence of the rapid clearance of subsequently injected PEGylated liposomes [18,19].

Liposomal-Encapsulated Vitamin C

Ascorbic acid (vitamin C) is a vitamin supplement as well as antioxidant and is usually added to many types of food products. The high reactivity and poor stability of vitamin C in solution impairs the role of vitamin C as part of the vitamin C is lost during food processing. Degradation of vitamin C in food has also been observed especially in the presence of oxygen or by anaerobic conditions in strongly acidic conditions. Encapsulating vitamin C into liposomes protects vitamin C from rapid destruction in food products as well as protecting the vitamin C from interacting with other food ingredients [20]. For optimum ascorbate protection, specific liposomes should be chosen and prepared. Liposomes made by the dehydration/rehydration method are efficient in protecting vitamin C at high concentrations of the nutrient. The presence of cholesterol in the liposomes will also help protect the encapsulated vitamin C as cholesterol will decrease the leakage of vitamin C from the liposomes during their circulation in the blood [17]. Nanoliposomes are also effective for the encapsulation

and controlled release of vitamin C as well as increasing its stability and bioavailability. Liposomes prepared with milk phospholipids using microfluidization were evaluated for vitamin C encapsulation. Increasing the number of passes through the micofluidizer resulted in a decrease in the size of the phospholipid liposome. The liposomes formed in the presence of vitamin C looked like empty liposomes in terms of size and lamellarity.

Entrapment of vitamin C increased when the phospholipids concentration also increased. Yet at high concentrations of phospholipids, no further vitamin C entrapment was observed. More research is still needed for liposomes prepared from phospholipids in terms of their stability and encapsulation ability [21]. In an *in vivo* study, the pharmacokinetics of phosphatidylcholine liposomal vitamin C formulations were compared to standard commercial preparations of vitamin C. All formulations contained 1gram vitamin C. The preliminary investigation showed that the liposomal formulation gave plasma ascorbate levels above 400 μML after large oral doses, while the standard vitamin C formulations gave ascorbate values above 220 μML. The findings of the study also showed that the absorption of vitamin C is not saturable at high doses as previously reported [22]. In another study conducted to evaluate the effect of phosphatidylcholine liposomal formulations on the bioavailability of vitamin C, the new findings showed that liposomal formulation gave higher circulating plasma levels of vitamin C compared to conventional oral deliver but less than the levels provided by intravenous vitamin C delivery yet the liposomal formulation gave similar protection against ischemia compared to intravenous administration vitamin C. Thus the bioavailability and efficacy of vitamin C increased when using liposomal formulations without the risk of giving vitamin C intravenously [23]. Vitamin C is also known as an antioxidant for the skin.

Yet oral summplementation of vitamin C does not increase the vitamin C skin concentration sufficiently. Topical delivery of vitamin C seems to be a good alternative. Various strategies have been investigated. Phosphatidylcholine liposomes have been investigated as a carrier to increase topical vitamin C delivery. The liposomal formulation allows stabilization of the ascorbate and allows targeting the deeper skin layers so that the vitamin C can give its beneficial effects. Vitamin C is shown to have antioxidant as well as anti-inflammatory properties against UVA/UVB photo-damage [24].

Liposomal Encapsulated Vitamin D

Vitamin D is a sterol compound which is the main part of cartilage and bone matrix in the human body. Vitamin D deficiency often occurs in countries where sunshine periods are short resulting in lower UVB radiation delivery especially during winter. Vitamin D deficiency can also occur in regions where there is sufficient sunshine due to the dress code required that covers a large amount of skin. In order to decrease such deficiency, supplements and diets containing vitamin D3 are very important. Vitamin D3 cannot dissolve completely in aqueous solutions due to its low polarity and susceptibility to oxidation as it is a lipid soluble vitamin. Encapsulation of vitamin D3 will protect it from oxidation during storage thus leading to increasing its effectiveness. Encapsulation also offers other advantages such as controlled release in nano or micro capsules. Nanocarriers will also increase the surface area increasing the solubility and bioavailability of vitamin D3 and targeting of the ingredients compared to microencapsulation. Lipid based nanocarriers for encapsulation stabilize such reactive materials. Liposomes and nanoliposomes are examples of such lipid based nanocarriers for vitamin D3 supplements. Vitamin D3 loaded nanoliposomes can be prepared via the thin film hydration-sonication method. Vitamin D3 is entirely encapsulated in the liposomes (almost 93%) as it is a lipophilic compound and is encapsulated in the liposomal bilayer. Cholesterol is added to the liposomal membrane to enhance liposomal stability in biological fluids such as blood and enhance the viscosity of such formulations. No significant changes in zeta potential of vitamin D3 encapsulated nanoliposomes usually occur indicating that such formulations are relatively stable [25]. Liposomes containing cholesterol in their bilayer are good models that can help the formation of vitamin D3 in the skin *in vivo* [26].

Liposomal Nanotechnology in Sport and Exercise Nutrition

The beneficial effect of vitamin C to improve sport and exercise performance has been extensively researched as atheletes are prone to upper respiratory tract infections due to physiological stress during training. Liposomal technology is a promising tool that could address the absorption and bioavailability of nutrients to the body. More research is still needed regarding the relationship between the use of liposomal technology in sport and exercise [27].

Biological Challenges Facing Liposomal Drug Delivery Systems

As with any foreign particle that enters the body, liposomes encounter multiple defense systems aimed at recognition, neutralization, and elimination of invading substances. These defenses include RES, oposonization, and immunogenicity. While these obstacles must be circumvented for optimal liposomal function, other factors such as the enhanced permeability and retention (EPR) effect can be exploited to enhance drug delivery [1].

Immunogenicity Testing

Immunogenicity testing of liposomes are becoming an important part of the drug development process as both manufacturers and regulatory agencies are considering immunogenicity testing as a way to determine whether patients are producing antibodies to biologics that can block the efficacy of the drugs. The European Medicines Agency (EMEA) issued a draft in 2007 on immunogenicity assessment which became final in April 2008, and the US FDA then put Assay Development for Immunogenicity Testing on its Guidance Agenda for 2008.

Immunogenicity testing takes place in three major steps: the screening assay, the confirmatory assay, and the neutralizing assay. Before these three major steps, an initial step is the characterization assay, which is of a research nature and usually performed on a small number of samples. The characterization assay determines which of the drug's epitopes binds to the antibody and the binding constants. The fact that immunogenicity responses to biologics cannot be expected to show up in animal models makes this testing crucial [28]. The screening assay is performed to see if patients taking the biologic are producing antibodies that bind to the drug. Patient serum is tested in the presence of the drug using an Enzyme-linked ImmunoSorbent Assay (ELISA) format or a similar technology. A typical assay for screening might be a bridge ELISA where antibodies that stick to the drug delivery system "bridge" two molecules of the drug delivery system.

First, patient serum, which may contain antibodies that bind to the drug delivery system, is incubated along with conjugate-labeled drug delivery system in a polypropylene plate. The serum-drug mixture is next transferred to a strepavidin-blocked plate; the conjugate-label enables the drug to be detected by laboratory equipment (the conjugate-label could be color or light emitting). The serum-drug mixture is next is next transferred to a strepavidin-blocked plate for an appropriate incubation time. The plates are then washed so that the antibodies that remain are those that are bound to the drug that is attached to the plate (the other wash away). The conjugate label enables laboratory instrumentation to detect the bound drug delivery system -antibody-drug delivery system that characterizes a bridge ELISA [28]. Patients samples that produce signals above a certain cut point may be subjected to a confirmatory assay. The screening assay is designed to minimize false negatives, so positive screening assays need to be confirmed as positive. These are usually performed using the same format as in the screening assay. A comparison of patient serum in the presence and absence of excess drug delivery system is taken to confirm or deny the existence of antibodies [28]. The third step is the neutralizing assay which tests to see whether the antibody binds to the drug delivery system in a way that can block the effectiveness of the drug. Living cells are often used in these assays to see whether the antibody is changing the biological process by which the drug acts [28].

The need for immunogenicity testing is growing as more and more drug delivery systems make their way into the drug development pipeline. Many of these drugs require immunogenicity testing after the drug in on the market as well [28]. Nonviral, lipid-based drug delivery systems are generally thought to be nonimmunogenic with respect to the components of the carrier [18]. Yet therapeutically relevant liposomes are recognized by the immune system and produce an immune reaction. Immune reactions that are initiated due to liposomal formulations can be stimulatory or inhibitory, weak, moderate or severe. Stimulation of immune response include hypersentivity to the liposomal formulation as well as producing antigens against such formulations. The stimulatory immune response may be immediate occurring within seconds or minutes and remaining for minutes or hours, delayed occurring within hours and remaining for minutes to hours, and late occurring within days to months and remaining from weeks to years. While the inhibitory immune response may be short-term remaining from hours to days or long-term remaining days to months. Two main reasons for the immunological response initiated by the liposomal formulations are:

1. The vesicle diameter between 50-200nm, and
2. Absence of cell membrane structures that prevent the host cells from recognizing the liposomal structures [29].

Conclusion

Liposomes have been investigated as nanocarriers to target drugs to their site of action for a long time. They have been explored as drug delivery systems due to their unique structure which is similar to the structure of cellular phospholipids and the fact that liposomes can be formulated in different forms. Liposomes have improved therapies for a range of biomedical applications by stabilizing therapeutic compounds, overcoming obstacles to cellular and tissue uptake, and improving biodistribution of compounds to target sties *in vivo*. The main obstacle in liposomal formulations are their ability to produce an immune response in some cases. Clinical trials and safety assessment remain vital in order to be able to use these formulations due to their extensive use.

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