MINIREVIEW

Trends and Developments in Liposome Drug Delivery Systems

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ABSTRACT: Since the discovery of liposomes or lipid vesicles derived from self-forming enclosed lipid bilayers upon hydration, liposome drug delivery systems have played a significant role in formulation of potent drugs to improve therapeutics. Currently, most of these liposome formulations are designed to reduce toxicity and to some extent increase accumulation at the target site(s) in a number of clinical applications. The current pharmaceutical preparations of liposome-based therapeutics stem from our understanding of lipid–drug interactions and liposome disposition mechanisms including the inhibition of rapid clearance of liposomes by controlling size, charge, and surface hydration. The insight gained from clinical use of liposome drug delivery systems can now be integrated to design liposomes targeted to tissues and cells with or without expression of target recognition molecules on liposome membranes. Enhanced safety and heightened efficacy have been achieved for a wide range of drug classes, including antitumor agents, antivirals, antifungals, antimicrobials, vaccines, and gene therapeutics. Additional refinements of biomembrane sensors and liposome delivery systems that are effective in the presence of other membrane-bound proteins *in vivo* may permit selective delivery of therapeutic compounds to selected intracellular target areas. J Pharm Sci 90:667–680, 2001

INTRODUCTION

Since the discovery in the 1960s that hydration of dry lipid film formed enclosed spherical vesicles or liposomes that resemble miniature cellular organelles with lipid bilayers,[1] the potential use of liposomes as biodegradable or biocompatible drug carriers to enhance the potency and reduce the toxicity of therapeutics was recognized. However, the application of liposomes to drug delivery systems was not realized until 30 years later. Only then were the first series of liposome-based therapeutics approved for human used by the U.S. Food and Drug Administration (FDA). Liposomes or lipid vesicles are colloidal particles that can be prepared with (phospho)lipid molecules derived from either natural sources or chemical synthesis. In the 1960s and 1970s, various liposome preparation methods were developed to study biological processes of membranes and membrane-bound proteins. By 1970, liposomes were proposed as drug carriers to modify the therapeutic index of a drug by reducing toxicity or increasing efficacy (or both) of the parent drug. Early research in liposomal drug preparations was beset with problems—there was insufficient understanding of liposome disposition and clear-

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Table 1. Liposome and Lipid-Based Products Approved for Clinical Use in the United States

Product	Drug (lipid:drug ratio)	Lipid Formulation	Marketed By	Indication
Doxil™ Caelyx™	Doxorubicin (8:1)	PEG-DSPE: HSPC: Cholesterol (5:56:39)	Alza Corporation (formerly, Sequus)	Kaposi sarcoma in AIDS Refractory ovarian cancer
DaunoXome™	Daunorubicin citrate (15:1)	DSPC: Cholesterol (2:1)	Gilead Sciences (formerly, NeXstar)	Kaposi sarcoma in AIDS
Ambisome™	Amphotericin B (3.8:0.4)	HSPC:DSPG: Cholesterol (2:0.8:1)	Gilead Sciences (formerly, NeXstar)	Serious fungal infections Cryptococcal meningitis in patients HIV^+
Amphotec™	Amphotericin B (1:1)	Cholesteryl sulfate	Alza Corporation (formerly, Sequus)	Serious fungal infections
Abelect™	Amphotericin B (1:1)	Lipid complex DMPC:DMPG (7:3)	Elan Corporation (formerly, The Liposome Company)	Serious fungal infections

Table 2. Liposome and Lipid-Based Products in Clinical Trials in the United States

Product	Drug	Formulation	Developed By	Status	Indication Sought
Allovectin-7	HLA-B7 plasmid	DNA-lipid complex (intralesional injection)	Vical Inc.	Phase II	Gene therapy of metastatic cancers
				Phase I	Gene therapy of metastatic renal cancer with concurrent IL-2
Annamycin	Annamycin	Liposomes	Aronex Pharmaceuticals	Phase I/II	Breast cancer
Antragen™	Tretinoin	Liposomes	Aronex, Pharmaceuticals	Phase II/III	Kaposi's sarcoma in AIDS
				Phase II	Recurrent acute promyelocytic leukemia
				Phase I	Cancer of blood
Nyotran™	Nystatin	Liposomes	Aronex Pharmaceuticals	Phase II/III	Candidemia
				Phase I	Comparative study against Amphotericin B in suspected fungal infection
TLC-D99 Evacet™ Mycocet™	Doxorubicin	Liposomes	Elan Corporation	NDA filed	Metastatic breast cancer
Ventus™	Prostaglandin E1	Liposomes	Elan Corporation	Phase III	Acute respiratory distress syndrome

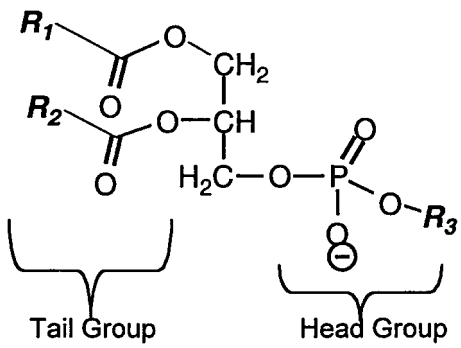
ance *in vivo*, inaccurate extrapolation of *in vitro* liposome–cell interactions or liposome targeting data, and insufficient stability and circulation time of liposome-based drugs *in vivo*. Advances in the late 1980s and early 1990s, including a detailed understanding of lipid polymorphisms, physiological mechanisms of *in vivo* liposome disposition, and lipid–drug and lipid–protein

interactions, overcame many of the early disappointments. The result was liposome designs with increased stability both *in vitro* and *in vivo*, improved biodistribution, and optimized resident time of liposomes in systemic (or blood) circulation. The goal of using liposomes as drug carriers in pharmaceutical applications was realized in the mid-1990s. A list of FDA-approved liposome-formulated drugs and those that are in current clinical trials are presented in Tables 1 and 2. The current pharmaceuticals in the United States that are formulated as liposome drug delivery systems are mainly antifungal and anticancer therapies; many more products, including those used as analgesics, gene therapies, and vaccines, are being developed. Because there are a number of recent and excellent review articles on the biophysical aspects of liposome preparation, characterization, and optimization,[2,3] basic properties of liposomes are only briefly discussed here. Recent progress and the insight gained from clinical use of liposome-formulated drugs is highlighted.

BASIC PROPERTIES OF LIPOSOMES

Liposomes or lipid vesicles are colloidal particles composed of (phospho)lipid molecules as the major constituent in formation of enclosed lipid bilayers or lipid–drug sheet–disk complexes. Although the lipid constituent can vary, many formulations use synthetic products of natural phospholipid, mainly phosphatidylcholine. A list of various phospholipids and their derivatives and intended applications is presented in Table 3.

Table 3. Commonly Used Phospholipids and the Attributes of Head and Fatty Acyl (Tail) Groups



Domain	Effect on Liposome Membrane	Functional Attribute on Lipid Bilayer
Tail group-Fatty acyl chains: R_1 and R_2 (C14-18 in length)		
Increase degree of saturation	Increase rigidity; decrease fluidity	Elevate T_c
Increase chain length of R_1 and R_2	Increase thickness of bilayer	Elevate T_c
Varying degree of saturation and chain length on R_1 and R_2	Decrease order of membrane packing	Lower T_c (compared to phospholipid with two identical fatty acyl tails)
Head group:R_3		
Choline: $–CH_2–CH_2–N(CH_3)_3^+$	Some surface hydration	Neutral charge
Ethanolamine:$–CH_2–CH_2–NH_3^+$	Mimimum degree of surface hydration	Neutral charge
Serine:$–CH_2–CH(COO^-)–NH_3^+$	Some surface hydration	Negative charge
Glycerol:$–CH_2–C(OH)CH_2OH$	Some surface hydration	Negative charge
PEG (ethanolamine): $–CH_2–CH_2–NH–PEG$	Enhanced surface hydration and steric effect	Negative charge

Most of the liposome formulations approved for human use contain phosphatidylcholine (neutral charge), with fatty acyl chains of varying lengths and degrees of saturation, as a major membrane building block (Table 1). A fraction of cholesterol (~30 mol%) is often included in the lipid formulation to modulate rigidity and to reduce serum-induced instability caused by the binding of serum protein to the liposome membrane. Cellular and physiological mechanisms explain the variations of liposome size, charge, surface hydration, membrane fluidity, and clearance of lipid-associated drug. Physical characteristics that determine liposome stability in storage and disposition *in vivo* (in particular, plasma clearance, *CL*) are some of the most important parameters for parenteral preparations of liposome-based therapeutics.

Surface Charge

Based on the head group composition of the lipid and pH, liposomes may bear a negative, neutral, or positive charge on their surface. The nature and density of charge on the surface of the liposomes influences stability, kinetics, and extent of biodistribution, as well as interaction with and uptake of liposomes by target cells. Liposomes with a neutral surface charge have a lower tendency to be cleared by cells of the reticuloendothelial system (RES) after systemic administration and the highest tendency to aggregate. Although negatively charged liposomes reduce aggregation and have increased stability in suspension, their nonspecific cellular uptake is increased *in vivo*. Negatively charged liposomes containing phosphatidylserine (PS) or phosphatidylglycerol (PG) were observed to be endocytosed at a faster rate and to a greater extent than neutral liposomes.[4,5] Negative surface charge is recognized by receptors found on a variety of cells, including macrophages.[4,6] Inclusion of some glycolipids, such as the ganglioside GM1 or phosphotidylinositol (PI), inhibits uptake by macrophages and RES cells and results in longer circulation times. It has been suggested that a small amount of negatively charged lipids stabilize neutral liposomes against an aggregation-dependent uptake mechanism.[3] Positively charged, cationic liposomes, often used as a DNA condensation reagent for intracellular DNA delivery in gene therapy, have a high tendency to interact with serum proteins; this interaction results in enhanced uptake by the RES and eventual clearance by the lung, liver, or spleen. This mechanism of RES clearance partly explains the low *in vivo* transfection efficiency. Other factors, including DNA instability, immune-mediated clearance, inflammatory response, and tissue accessibility may also contribute to low transfection efficiency in animals. In fact, high doses of positively charged liposomes have been shown to produce varying degrees of tissue inflammation.[7]

Surface Hydration or Steric Effect

The surface of the liposome membrane can be modified to reduce aggregation and avoid recognition by RES using hydrophilic polymers. This strategy is often referred to as surface hydration or steric modification. Surface modification is often done by incorporating gangliosides, such as GM1 or lipids that are chemically conjugated to hygroscopic or hydrophilic polymers, usually poly(ethyleneglycol) (PEG). This technology is similar to protein PEGylation. Instead of conjugating PEG to therapeutic proteins such as adenosine deaminase (Alderase, for treatment of severe combined immunodeficiency syndrome) to reduce immune recognition and rapid clearance,[8] PEG is conjugated to the terminal amine of phosphatidylethanolamine. This added presence of hydrophilic polymers on the liposome membrane surface provides an additional surface hydration layer.[9] The resulting liposomes cannot be recognized by macrophages and RES as foreign particles, and are spared phagocytic clearance. A number of systematic studies have determined the optimum size of PEG polymer and the density of the respective polymeric PEG lipid in the liposome membrane. The optimum size of PEG is $MW_{avg} = 1–2000$[10] and the presence of PEG–lipid at 5–10 mol% total lipid composition; under these conditions, liposomes can encase both lipophilic and hydrophilic molecules in a stable manner, while avoiding RES uptake. Depending on the length of the PEG polymer, PEG on the liposome membranes occupies an additional 5 nm of surface hydration thickness[11] without significantly modifying the overall charge property of liposome membranes. One of the key advantages of using PEG-conjugated lipid is the long-standing human safety data on the use of PEG as excipient for parenteral preparations. However, heterogeneity of long-chain PEG polymers, purified from petroleum products, and the slow renal clearance of extremely large PEG polymers may be concerns.

Other amphiphilic polymers with similar properties, such as poly(acryloyl)morpholine (PacM), poly(acrylamide) (PAA), and poly(vinylpyrrolidone) (PVP), have also been conjugated to phospholipids and used as liposome steric protectors with varying degrees of success.[12] Their safety in humans is less well understood.

Fluidity of Lipid Bilayer

Lipid bilayers and liposome membranes exhibit a well-ordered or gel phase below the lipid phase transition temperature (T_c) and a disordered or fluid phase above the T_c. The lipid phase transition is measured and expressed as T_c, the temperature at which equal proportions of the two phases coexist. At a temperature corresponding to T_c, a maximum in liposome leakiness is observed.[13] The phase behavior of a liposome membrane determines permeability, aggregation, protein binding, and, to a lesser degree, fusion of liposomes. Because the T_c varies depending on the length and nature (saturated or unsaturated) of the fatty acid chains (Table 3), the fluidity of bilayers can be controlled by selection and combinations of lipids. For instance, incorporation of cholesterol at a low concentration into the bilayer leads to an increase in the transmembrane permeability, whereas incorporation of higher amounts (>30 mol%) of cholesterol can eliminate phase transition and decrease the membrane permeability at a temperature $>T_c$.[14] Various phase transitions of lipid bilayers have been designed to induce liposome fusion and drug release. Encapsulated drugs can be released into the target tissue by modulating local tissue temperature by external heating using various sources of energy, such as infrared, microwave, or laser light. However, drugs bound to lipid membranes or protein-bound lipid membranes may shift the transition temperature or abrogate the phase transition behavior all together.[15,16] Binding of serum proteins also influences the phase transition behavior and release of the aqueous contents of liposomes[17] In addition, fluidity, in particular liposomes that exhibit phase transition behavior at or near physiologic temperatures (~37 °C), may enhance phospholipase activity at the cell surface, generating lysophospholipids (by deacylation at A_1 or A_2 positions of phospholipids). Lysophospholipids in rat spinal tissues were shown to produce behavioral neurotoxicity in rats after intrathecal administration.[18]

Liposome Size

Early research has demonstrated that liposome size affects vesicle distribution and clearance after systemic administration. The rate of liposome uptake by RES increases with the size of the vesicles.[19] Whereas RES uptake *in vivo* can be saturated at high doses of liposomes or by predosing with large quantities of control liposomes, this strategy may not be practical for human use because of the adverse effects related to the impairment of RES physiological functions. The general trend for liposomes of similar composition is that increasing size results in rapid uptake by RES.[20] Most recent investigations have used unilamellar vesicles, 50–100 nm in size, for systemic drug delivery applications. For example, the antifungal liposome product AmBisome is formulated to the size specification of 45–80 nm to reduce RES uptake. Serum protein binding is an important factor that affects liposome size and increases the rate of clearance *in vivo*. Complement activation by liposomes and opsonization depend on the size of the liposomes.[21,22] Even with the inclusion of PEG in the liposome compositions to reduce serum protein binding to liposomes, the upper size limit of long-circulation PEG–PE liposomes is ~150–200 nm. Due to biological constraints, development of long circulating large (>500 nm) liposomes using steric stabilization methods has not been successful. Hence, considerations of liposome size and its control in manufacturing at an early stage of drug development provides a means to optimize efficiency of liposome drug delivery systems.

DISPOSITION OF LIPOSOMES *IN VIVO*: INSIGHT GAINED FROM PRECLINICAL AND CLINICAL STUDIES

The exact mechanisms of biodistribution and disposition *in vivo* vary depending on the lipid composition, size, charge, and degree of surface hydration/steric hindrance. In addition, the route of administration may also influence the *in vivo* disposition of liposomes. Immediately after intravenous administration, liposomes are usually coated with serum proteins and taken up by cells of RES and eventually eliminated.[23,24] Plasma proteins that may interact with liposomes include albumin, lipoproteins [i.e., high-density lipoprotein (HDL), low-density lipoprotein (LDL), etc.] and other cell-associated proteins. Some of these

proteins (e.g., HDL) may remove phospholipids in the liposome bilayer, thereby destabilizing the liposomes. This process may potentially lead to a premature leakage or dissociation of drugs from liposomes. In addition, in the case of acid or pH-sensitive liposomes, protein binding may abrogate the pH sensitivity of liposomes. Lipid–protein interactions may also explain the drastically reduced transfection activity of DNA–cationic lipid complex *in vivo*. In addition, plasma protein binding has also been shown to modify the gel-to-liquid phase transition of phospholipids with a saturated fatty acyl chain, such as Dipalmitoylphosphatidylcholine (DPPC) ($T_c = 37$ °C).[17] In addition to modifying the drug release from liposomes, protein binding may also lead to immunologic consequences, such as complement activation due to the nonspecific cationic lipid binding as observed in mice.[25] Whether complement activation is a significant issue in delivery of DNA in humans with cationic lipids remains to be studied.

Although the liposomes coated with hydrophilic polymers such as PEG reduce the RES-mediated clearance, all the liposomes are eventually cleared to liver and spleen.[26] A small faction of liposomes may distribute to the site of infection or where rapid tumor growth occurs. Also, a small fraction of liposomes may distribute to skin and extremities and clear from these tissues at a much slower rate. Although enhanced localization of liposomes (e.g., those containing doxorubicin or daunorubicin) to skin may provide therapeutic benefits for AIDS patients with Kaposi's sarcoma, it may also produce dermal lesions often referred to as hand and foot syndrome (Palmer-plantar erythrodysesthesia syndrome).[27] It has been proposed that infection and tumor growth induce inflammation, which compromises the vasculature permeability, and thereby enhances the accumulation of liposome-associated or encapsulated drugs to these sites.[28] If this liposome accumulation mechanism is in operation, the increased circulation time that results from preventing "first-pass" hepatic clearance of liposomes will provide a higher degree of liposome-associated drug in the target (e.g., tumors or infection) sites.

Subcutaneously and intramuscularly administered large liposomes may be trapped at injection sites and serve as a drug depot.[29] On the other hand, small (50–80 nm) liposomes administered subcutaneously will be retained in draining lymph nodes and eventually redistribute drugs into blood circulation. The mechanism of enhanced liposome localization is due to the particle size limitation of lymph node drainage.[30] Size-dependent latex and carbon particle studies estimated the upper size limit of lymph node drainage to be 20–30 nm.[31, 32] Hence, lipid–drug complexes >40–50 nm will likely be retained in the lymph node as it enters the lymphatic system.[33,34] Accumulation of liposomes in lymph nodes may allow us to enhance delivery of drugs to rapidly growing tumors in lymph nodes during the metastatic stage of a number of cancers, or reduce virus load of HIV-positive patients. Despite the use of combination antiviral drug therapies, virus burden in lymph nodes of HIV patients remains relatively high,[35] and such an enhanced antiviral delivery strategy may provide a high anti-HIV drug concentration in lymph nodes with an acceptable margin of safety.

Next, we will briefly discuss the collective experience with using long-circulating liposomes; a discussion of active targeting of liposomes with respective ligands or receptors expressed on liposome surface follows.

Passive Targeting of Liposomes with Prolonged Resident Time in Blood

One of the key properties that make liposomes an invaluable drug delivery system is their ability to modulate pharmacokinetics of liposome-associated and encapsulated drugs.[4,19,36] Relative to the same drugs in aqueous solution, significant changes in absorption, biodistribution, and clearance of liposome-associated drug are apparent, resulting in dramatic effects on both the efficacy and toxicity of the entrapped compound.[37,38] But therapeutic applications of systemically administered liposomes have been limited by their rapid clearance from the bloodstream and their uptake by RES in liver and spleen.[39]

As already mentioned, circulation time can be increased by reducing liposome size and modifying the surface/steric effect with PEG derivatives. Engineered liposome membranes with sufficient stability that also escape clearance by RES are now available. Therefore, long-circulation liposomes that also significantly reduce toxicological profiles of respective drugs can be used to maintain and extend plasma drug levels. Even though only a small fraction of liposomes eventually accumulate at target sites, prolonged circulation can indirectly enhance accumulation of liposome-associated drugs to targeted tissues.

Table 4. Selected List of Ligands and Receptors Tested as Liposome-Targeting Agents

Molecule	Ligand/Receptor	Target Cell/Tissue	Reference
Antibodies			
H1817	E-selectin	human umbilical vein endothelial cells	72
CD19	CD19	B cell lymphoma	73
CD35	CD35	hematopoietic progenitors	74
N-12A5	erbB-2(Her/2)	tumor cells	75
Anti-HLA-DR	HLA-DR (MHC-II)	lymph nodes	76
Anti-Selectin		inflammatory sites	77
G-22	glioma-associated antigen	glioma cells	78
Transferrin	transferrin receptor rat glioma cells		
Proteins or peptides			
HIV-gp 120	CD4	CD4 cells	79
HIV-gp 120 peptides	CD4	HIV-infected cells	80
Cytokine			
IL2	IL2 receptor	T cells	81
Growth Factors			
Transferrin	Transferrin receptor	tumor cells/tumor	52
Beta nerve growth factor (NGF)		NGF receptor	82
Hexose and derivatives			
Galactoside	Asialoglycoprotein receptor	hepatocyte	83
Galactosylated histone	Asialoglycoprotein receptor	hepatocyte	84, 85
Asialofetuin (AF)	Asialoglycoprotein receptor	hepatocyte	85
Other			
Apo E/Apo B	LDL receptor	tumor cells	49
Folate	folate receptor	tumor cells	86
Fibrinogen	fibrin/thrombi	fibrous atheroma thrombi	87
ICAM-1	ICAM-1	atherosclerotic	88
Cholera toxin B subunit	GM-1	mouse epithelial cells and tissues	89, 90

Active Targeting of Liposomes

For certain drug delivery applications requiring rapid responses, an active delivery system that can facilitate binding to a selective cell type within a given tissue may be required.

Since the discovery of liposome applications, it has been a goal for many scientists to develop liposomes that can target specific cells using ligands or receptors that are unique to the target tissue or cells. A majority of active liposome target delivery systems use chemically coupled ligands expressed on liposome membranes. Using this strategy, a variety of ligands or receptors, such as antibodies, growth factors, cytokines, hormones, and toxins, have been anchored and expressed on liposome surfaces so that drugs, proteins, and nucleic acids may be introduced into target cells (Table 4). *In vitro*, liposomes coated with monoclonal antibodies (immunoliposomes) can provide target-specific binding to cells.[40] However, a number of key issues must be addressed before active targeting of liposomes using ligand–receptor interactions can be realized *in vivo*. (1) The liposomes expressing specific targeting molecules must circulate in blood long enough to localize and perfuse into the target organ or tissue and eventually interact with and bind to the cells. Without sufficient resident time in systemic circulation, most of the liposomes will be cleared without making contact with the cell targets. (2) The ligand or receptor on the target cells must provide sufficient specificity. A number of cell-associated ligands, receptors (i.e., galactose receptors on hepatocytes or folate receptors in actively growing cells and tumors), or antigens [i.e.,

carcino-embryonic-antigen (CEA)] can be used for targeting. But there are many other nontargeted normal cells that express these receptors in a low density. If fact, it has been proposed that there is no tissue- or tumor-specific antigen; rather, the respective tumor-associated antigen or ligands are expressed at much lower levels in normal cells. Therefore, some knowledge of the density of the receptor in target tissue compared with nontargeted sites and the degree of blood perfusion will be useful to refine the design of targeted liposome formulations. (3) In addition, the targeting molecule(s) expressed on the liposome surface must be sufficiently stable *in vivo* and exhibit minimum potential of being removed by serum proteins.

The elucidation of physiological liposome disposition mechanisms and the development of long-circulation liposomes have narrowed the gap in achieving the goal of target-specific liposome drug delivery systems. Initial targeting experiments using antibody expressed on PEG-coated liposomes demonstrated that PEG may interfere with target cell binding.[9,41]Additional studies suggested that PEG length is crucial for immunoliposomes. To overcome the barrier of PEG, attachment of antibody to the distal top of a PEG molecule has been shown to provide specificity for liposome binding to cells *in vitro* that express the respective receptor (antigen) on their surface.[36,42] Similar methods have been used successfully to bind liposomes to tissue in the lung[43] and brain[44] *in vivo*. The PEG–PE liposomes with antibodies retain long survival times in the circulation and demonstrate target recognition *in vivo*.[45,46] A number of methods for coupling mAb at the PEG terminus have been developed recently.[36,47] However, sterically stabilized liposomes with antibody seem to lose their advantage in treating advanced solid tumor because of limited perfusion to the tumor interior.[48]

In addition to antibodies, other ligands, such as apo-E (glycoprotein apolipoprotein E), which is a high-affinity ligand for the LDL receptor, can be incorporated into small liposomes to target tumor cells that express a high density of LDL receptors.[49] Because folic acid receptors are overexpressed in human cancer cells,[50] folic acid anchored on liposomes increases uptake and internalization of liposomes by tumor cells.[6,51] Knowing that actively growing tumor cells express a high density of transferrin receptors (for ion uptake) led to the development of a number of strategies to express transferrin on liposomes and enhanced delivery of anthracyclines, such as adriamycin, and methotrexate to tumor cell lines *in vitro*.[52] Whether there will be a significant improvement in controlling tumor growth *in vivo* using these strategies remains to be seen.

Although these approaches in anchoring the antibody, its derivatives, and ligands on the liposome surface may provide target selectivity *in vitro* and *in vivo*, the cost and reproducibility of these derivatives in quality and quantity sufficient for pharmaceutical applications are challenging problems. The lipopeptides approach (i.e., attaching an acylated peptide onto the liposome surface) may produce more efficient and reproducible outcomes.[53] However, large-scale synthesis of lipopolypeptides may be a costly venture. Recently, a single-chain, minimum-binding domain of antibody (Ig) molecule, scFv (MW = 25–27 kDa), was cloned and successfully produced by a recombinant bacteria fermentation process in a cost-effective manner.[54] The prospect of using the recombinant monoclonal antibody derivatives, scFv, anchored on the liposome surface to target a wide range of tissues and cells for therapeutic applications may become practical. Theoretically, amino acids prone to be acylated by post-translational modification in a bacteria host can be inserted in the DNA sequences linking the hypervariable regions of FV molecules to produce recombinant scFV products that are acylated. These acylated scFVs can be readily incorporated into liposomes with ease and efficiency required for a successful pharmaceutical scale preparation. scFVs with specificity to a wide range of target antigens, including a number of tumor-associated antigens, vesicular endothelial cell growth factors (e.g., VEGEF, endostatin, etc.),[55] and T cell receptors (CD3, CD34, etc.), can be engineered by high throughput screening systems, including phage display expression technology.[56] Incorporation of acylated antibodies and peptides has been well characterized and proven to be an efficient process.[40,57]

Although target delivery of liposomes has shown promising results *in vitro*, *in vivo* and clinical data must be collected. In coupling ligands to liposomes, we should consider their size, coupling methods, and binding features. Ligand–receptor specific liposome delivery can only be achieved *in vivo* when a significant fraction of liposomes is perfused into the tissue, allowing the interactions of targeted liposomes with their respective ligand or receptors on target cells. Only then will efficiency of liposome-

mediated delivery become dependent on the ligand–receptor affinity. The practicality for a large-scale pharmaceutical preparation of targeted liposomes must be considered early in the drug design and development process to further advance the clinical use of liposome drug delivery systems to significantly improve the safety and efficacy of highly potent therapeutic compounds.

OTHER APPLICATIONS

Vaccine Delivery

The mechanism by which liposomes enhance antigen-specific immune response is not fully understood. From *in vivo* liposome disposition studies, it is clear that large liposomes are taken up efficiently by macrophages of RES in blood and tissues, including the liver and spleen. Because macrophages are thought to be predominantly responsible for processing and presenting liposome-associated and encapsulated antigens, the liposome formulation provides an excellent way to enhance antigen delivery and presentation for both humoral and cellular immune stimulation of vaccines. Some of these results have been reviewed.[24] In addition to macrophages that predominantly present antigen in the context of major histocompatibility antigen (MHC) class II antigen (for antigen-specific B cell growth or antibody production), antigen delivered to some of the endothelial, Langerhan's, and dendritic cells may enhance the antigen presentation in the context of MHC class I antigen in mediating cellular immune responses, including cytotoxic T cell responses.[58]

The influence of physicochemical properties of the liposomes, such as charge density, membrane fluidity, and epitope density, on the immune response of the antigen has been extensively studied.[59] In addition to antigen, other immune stimulators that are amphiphilic muramyl peptides or lipid-soluble, such as monophosphoryl lipid A and muramyl tripeptidyl-phosphatidylethanolamine, can also be incorporated into liposomes to increase their adjuvant effect.[60]

Recently, the new liposome-based hepatitis A vaccine (Epaxal®) developed by Swiss Serum and Vaccine Institute has been tested in humans.[61] This vaccine contains formalin-inactivated hepatitis A virus particles attached to phospholipid vesicles together with influenza virus hemaglutinin. The advantage of such a vaccine is that it not only induces antibody to hepatitis A antigen but also influenza virus protein expressed on the liposome surface.[62] The mechanisms of liposome-mediated immune enhancement, however, are quite complex.

Gene Therapeutics

Although a number of cationic lipids and other cationic polymeric compounds are used as DNA condensation agents to enhance delivery of DNA plasmid in gene therapy, the experience to date suggests that none of these agents appear to significantly and consistently improve the expression of DNA delivered by cationic lipids compared with the results of free, naked plasmid DNA injection. Although intravenous administration of DNA–lipid complexes may reduce the degradation rate of DNA *in vivo*, the degree of protein binding of complement to cationic components of lipids may reduce transfection efficiency, and in some cases, result in complement activation.

A number of recent review articles focused on these topics.[63–65] Initial series of cationic constructs uses a linear positive charge on the head group of molecules that resemble lipids or cholesterol for condensation of DNA, with the inclusion of an unsaturated phosphatidylethanolamine (i.e., dioleoylphosphatidylethanolamine) to enhance intracellular (cytoplasmic) release of lipid–DNA complexes. Using a spermine, "T-shape", positively charged head group structure that anchored to liposome via cholesterol, [i.e., 3-β-(N^4-spermine carbonoyl)cholesterol (lipid #67 or GL-67)], the transfection efficiency of plasmid DNA can be improved by 100-fold compared with other cationic lipids with a linear positive charge head group. In a double-blind placebo-controlled trial of eight cystic fibrosis patients, the spermine positive charge lipid GL-67 enhanced transfection of plasmid containing cystic fibrosis transmembrane conductance regulator (CFTR) gene when delivered by nebulization to lung and nasal mucosa.[66] Limited efficacy was demonstrated with transcription of mRNA, changes in potential due to CFTR expression, chloride efflux function, and bacterial adherence. Whether this and other cationic lipids may provide enhanced systemic delivery of lipid–DNA complexes in serum remains untested.

The variable and untoward effects seen with cationic lipids may be less than the immune reactions mounted against DNA sequences delivered by viral vectors.[67] With recent discovery of the limitations of viral vectors such as adeno and

adeno-associated viruses,[68] nonviral-mediated gene transfer methods with lipid–DNA complexes may merit further exploration.

Oral Drug Delivery

The use of liposomes in oral drug delivery has been widely studied and reviewed recently.[69] Three major destabilizing factors are pH, bile salt, and pancreatic enzymes in the gastrointestinal (GI) tract. Several membrane surface polymerization chemistry methods have been developed to shield liposomes and their contents from the hostile environment of the GI tract; however, incomplete polymerization and toxicity of residual reagents and derivatives remain a concern. Alternatively, liposomes may be used as a solubilizing or suspension agent for highly insoluble or lipophilic drugs to be delivered as a microemulsion in softgel capsules for oral dosage. Microemulsion encased in softgel capsules was used to enhance reproducibility and bioavailability of cyclosporin A, originally formulated as a tablet.[70] An oral liposome–antigen formulation may also be used to stimulate mucosal immune responses, enhancing delivery of antigen to antigen-presenting cells that actively take up particles in the GI tract. Additional benefits of using liposomes for such applications include biocompatibility, flexbility in design, protection of antigen, targeting of antigens to antigen-presenting cells, and ineffective stimulation of immune responses by oral delivery of soluble antigens.[69]

FUTURE DIRECTIONS

Elucidation of physiological liposome disposition mechanisms have led to the design of small (~50 nm diameter) and sterically stabilized liposomes to increase their systemic resident time required for clinical applications. New development in molecular design for expression of ligand or receptor molecules on the surface of the liposome may improve the interaction of liposomes with cells. The improved liposome resident time (due to reduced CL) will provide liposome-associated drugs a chance to eventually reach their intended target sites. A first step to increase the intracellular uptake of liposomal drugs (anticancer agents, antibiotics, DNA) is to enhance its localization selectively within the target tissue. As additional ligands with higher affinity and specificity continue to be developed, and progress is made in antibody engineering to mass produce targeted liposome preparations, liposome–drug complexes with extended therapeutic indices are now within reach. Recently, HER2 antibody (binds to erb-2 oncogene products on select tumor cells) expressed on liposomes showed encouraging results in preclinical studies.[71] If these results can be confirmed in human trials, we may soon have targeted liposome delivery systems that can potentially be used to formulate high potency drugs with significantly improved safety and efficacy. Additional development of biomembrane sensors that function effectively (e.g., pH sensitivity for cytoplasmic delivery, nuclear membrane recognition for DNA delivery to nuclei) in an *in vivo* blood and tissue environment will add significantly to the success of delivering drug not only to cells, but also to selective organelles within the target cells, using liposome drug delivery systems.

GLOSSARY

FDA	United States Food and Drug Administration
CL	Clearance
RES	Cells of reticuloendothelial system
PS	Phosphatidylserine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PEG	Poly(ethyleneglycol)
PacM	Polyacryloylmorpholine
PAA	Polyacrylamide
PVP	Polyvinylpyrrolidone
T_c	Lipid phase transition temperature
HIV	Human immunodeficiency virus
LDL	Low density lipoprotein
HDL	High density lipoprotein
scFV	Single-chain minimum binding domain of an immunoglobulin molecule
MHC	Major histocompatibility antigen
CFTR gene	Cystic fibrosis transmembrane conductance regulator gene
PEG-DSPE	PEG-conjugated disteroyl-phosphatidylethanolamine
HSPC	Hydrogenated soy-derived phosphatidylcholine
DSPC	Disteroylphosphatidylcholine
DSPG	Disteroylphosphatidylglycerol
DMPG	Dimyristoylphosphatidylglycerol
DMPC	Dimyristoylphosphatidylcholine
DPPC	Dipalmitoylphosphatodylcholine

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