Review Article

Gene Therapy: Potential Use of Liposomes

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ABSTRACT

The primary challenge for gene therapy is to develop a method that delivers a therapeutic gene (transgene) to selected cells where proper gene expression can be achieved. Gene therapy using viral or synthetic vectors is currently one of the most promising strategies for many diseases. Cationic lipid–DNA complexes have emerged as one of the major non-viral DNA delivery tools. An ideal gene delivery method needs to meet 3 major criteria: (1) it should protect the transgene against degradation by nucleases in intercellular matrices, (2) it should bring the transgene across the plasma membrane and into the nucleus of target cells, and (3) it should have no detrimental effects.

Keywords: Gene therapy, gene expression, synthetic vectors, non-viral DNA delivery.

INTRODUCTION

Gene therapy aims at treating disease by genetically modifying populations of cells that are either directly functionally impaired or capable of relieving the disease symptoms. These genetic modifications can either increase or reduce the expression of specific genes or gene sets, or even restore the normal function of the product of these genes.[1] Crucial to the success of DNA as a pharmaceutical or a basic research tool is transfection efficiency: in general practice, too few cells receive and express the exogenous DNA. Efficiency of transfection is dependent on both the efficiency of DNA delivery (i.e., fraction of DNA molecules getting into the nucleus) and the efficiency of DNA expression (i.e., fraction of nuclear DNA molecules that undergo transcription).

Although a greater efficiency of expression can usually be achieved with strong promoters and enhancers, [2] improvements in the efficiency of DNA delivery per se have been difficult to achieve; thus, the number of cells receiving DNA in their nucleus is usually small. In addition, transfection efficiency in vitro and in vivo do not always correlate,[3] making translation of positive results in cell culture into animals even more difficult. Developing an efficient gene therapeutic approach and designing safe and efficient gene delivery reagents are inseparable. Shortcomings in one will adversely affect the success of the other.

Vectors for gene therapy

Transfection vectors commonly used in gene therapy are mainly of two types-viral and non-viral. *In vivo* gene transfer using viral vectors is today the most commonly used approach, with 20 trials listed in 2010.[4] This approach takes advantage of the viruses' ability to deliver their genetic material to target cells, including nondividing cells, and to induce long-term transgene expression. Much effort has been devoted to the development of non-viral delivery due to the disadvantages of viruses used for gene delivery. The disadvantages of viral delivery include generation of immune responses to expressed viral proteins that subsequently kill the target cells required to produce the therapeutic gene product, random integration of some viral vectors into the host chromosome, clearance of viral vectors delivered systemically, difficulties in engineering viral envelopes or capsids to achieve specific delivery to cells.[5] Methods of nonviral gene delivery have also been explored using physical (carrier-free gene delivery) and chemical approaches (synthetic vector-based gene delivery). Physical approaches employ a physical force e.g. needle injection, electroporation, gene gun, ultrasound and hydrodynamic delivery.[6-10] The chemical approaches use synthetic or naturally occurring compounds as carriers to deliver the transgene into cells.[11-12] Delivery of nucleic acids using liposomes holds great promise as a safe and non-immunogenic approach to gene therapy.

Liposomes exhibit several properties which may be useful in various applications. The most important properties are colloidal size, i.e. rather uniform particle size distributions in the range from 20 nm to 10 µm, and special membrane and surface characteristics. They include bilayer phase behaviour, its mechanical properties and permeability, charge density, presence of surface bound or grafted polymers, or attachment of special ligands, respectively. Additionally, due to their amphiphilic character, liposomes are a powerful solubilizing system for a wide range of compounds. In addition to these physico-chemical properties, liposomes exhibit many special biological characteristics, including (specific) interactions with biological membranes and various cells.[13]

Journey to the nucleus

Most DNA delivery systems operate at one of three general levels: DNA condensation and complexation, endocytosis, and nuclear targeting/ entry. Vectors for delivery must be able to (i) complex nucleic acids in stable, nanoscaled and positively charged aggregates, (ii) promote the internalization of DNA by cells, (iii) prevent the intracellular DNA degradation and, finally, (iv) induce exogenous gene expression.[14] Endocytosis is a multistep process involving binding, internalization, formation of endosomes, fusion with lysosomes, and lysis. The extremely low pH and enzymes within endosomes and lysosomes usually bring about degradation of entrapped DNA and associated complexes. Finally, DNA that has survived both endocytotic processing and cytoplasmic nucleases must then dissociate from the condensed complexes either before or after entering the nucleus. Entry is thought to occur through nuclear pores (which are ~10 nm in diameter) or during cell division. Once inside the nucleus, the transfection efficiency of delivered DNA is mostly dependent on the composition of the gene expression system.[3] The low efficiency of DNA delivery from outside the cell to inside the nucleus is a natural consequence of this multistep process. As a result, the number of DNA molecules decreases at each step of the journey to the nucleus. There are three major barriers to DNA delivery: low uptake across the plasma membrane, inadequate release of DNA molecules with limited stability, and lack of nuclear targeting.[3] Therefore, identifying and overcoming each hurdle along the DNA entry pathways can improve DNA delivery, and hence overall transfection efficiency.

LIPOSOMAL GENE THERAPY

What are liposomes?

Liposomes are vesicular structures that are formed due to accumulation of lipids interacting with one another in an energetically favorable manner. Depending upon the structure and the composition of the bulk solution, liposomes can separate hydrophobic or hydrophilic molecules from the solution. These vesicles are not rigid formations but rather are fluid entities that are versatile supramolecular assemblies. Because they have dynamic properties and are relatively easy to manipulate, liposomes have been used widely in the analytical sciences as well as for drug and gene delivery.[15]

Liposomes were described in 1965 and were soon proposed as drug delivery systems. For over almost 5 decades, various researchers have worked on liposomes which has led to the development of important technical advances such as remote drug loading, extrusion for homogeneous size, long-circulating (PEGylated) liposomes, triggered release liposomes, liposomes containing nucleic acid polymers, ligand-targeted liposomes and liposomes containing combinations of drugs.[16]

Advantages of liposomal gene therapy

The advantages in using liposomes for gene therapy are several and include the lack of immunogenicity after in vivo administration, lack of clearance by complement using improved formulations, unlimited size of nucleic acids that can be delivered (from single nucleotides to large mammalian artificial chromosomes), ability to perform repeated administrations in vivo without adverse consequences, low cost and relative ease in creating nucleic acid-liposome complexes in large scale for use in the clinic, relative ease in creating targeted complexes for delivery and gene expression in specific cell types, organs or tissues, and greater safety for patients due to few or no viral sequences present in nucleic acids used for delivery, thereby precluding generation of an infectious virus.[17,5]

CATIONIC LIPOSOMAL DELIVERY

Strategy behind cationic lipid mediated gene delivery

DNA being polyanionic macromolecule is not expected to be incorporated inside the cell as biological cell surface is negatively charged. The idea behind cationic lipid strategy is to neutralize the negative charge of plasmids with positively charged lipids to capture plasmids more efficiently and to deliver DNA into the cells.[18]

Liposomal vesicles have drawn the attention of researchers as potential carriers of various bioactive molecules that could be used for therapeutic applications in both humans and animals.[19-20] Recent work has shown that nucleic acids can be entrapped in cationic liposomes (CLs) and subsequently transfected into cultured mammalian cells, where they can express the information they carry. CLs represent one of the most widespread nonviral transfection systems for gene delivery. CLs are usually employed as a gene delivery system because of their low toxicity, low immunogenicity, ease of preparation, size-independent delivery of nucleic acids, and quality control and capacity for mass production at reasonable cost.[21] A solution of cationic lipids, often formed with neutral helper lipids, can be mixed with DNA to form a positively charged complex termed a lipoplex.[22] Well-characterized and widely used commercial reagents for cationic lipid transfection include N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA),[23] [1,2-bis(oleoyloxy)-3-(trimethylammonio) propane] (DOTAP),[24] 3β[N-(N´N´-dimethylaminoethane)-carbamoyl] cholesterol (DCChol),[25] and dioctadecylamidoglycylspermine (DOGS).[26] Table 1 lists various phospholipids used in liposome formation and their role in targeting particular disease.

Table 1: Lipid composition for liposomes and target disease

Drug	Liposome constituents	Therapy	Ref
Amphotericin B	Cholesteryl sulfate	Fungal Infections	31
Doxorubicin	HSPC, cholesterol, and PEG 2000-DSPE, Chloesterol	Kaposi's sarcoma, Ovarian/breast cancer	31-32
Cytarabine	Triolein, DOPC, and DPPG, Chloesterol	Neoplastic meningitis and lymphomatous meningitis	31
Inactivated hepatitis A virus (strain RG-SB)	DOPC and DOPE	Hepatitis A	33-34
Daunorubicin	DSPC and cholesterol	Blood tumors	35
Inactivated hemaglutinine of Influenza virus strains A and B	DOPC and DOPE	Influenza	36
Acid β-glucosidase	Phosphatidylcholine	Gaucher's disease	37
Doxorubicin	Palmitoleylphosphatidyl choline (POPC), DOPC-PEG, Chloesterol	Cancer	38
Chloroquine	Egg phosphatidylcholine (egg PC), Chloesterol	Malaria	39
AmphotericinB and nystatin	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine(DSPE)	Infectious diseases	40
Linoleic acid	phosphatidylcholine	Dermatology & cosmetology	41

Each cationic lipid has different structural aspects, such as head group size and hydrocarbon tail length. These aspects confer distinct characteristics to the lipid/DNA complex, which in turn affect association with and uptake into the cell. The positive charge on the head group facilitates spontaneous electrostatic interaction with DNA, as well as binding of the resulting lipoplexes to the negatively charged components of the cell membrane prior to cellular uptake.[27] Hydrophobic tails are not the only liposomal features that play a role in effective gene delivery—ionizable head groups are also involved. Some examples are the multivalent cationic lipids DOSPA and DOGS both of which have a functionalized spermine head group that confers the ability to act as a buffer, such as in the case where there is an influx of protons into a maturing endosome/endolysosome.[28] Such buffering could extend the amount of time needed to activate acid hydrolases and could explain why some multivalent cationic lipids can exhibit higher transfection efficiencies versus their monovalent counterparts.[29]

There is ongoing extensive research activity to overcome the major drawback of CL-vectors, which is their low transfection efficiency. For this purpose, numerous lipid formulations have been systematically studied. They typically contain a combination of cationic and neutral (helper) lipids such as DOPE or cholesterol.

Modifications for improved liposome-mediated gene delivery

Many cationic lipids show excellent transfection activity in cell culture, but most do not perform well in the presence of serum, and only a few are active in vivo.[12] A dramatic change in size, surface charge, and lipid composition occurs when lipoplexes are exposed to the overwhelming amount of negatively charged and often amphipathic proteins and polysaccharides that are present in blood, mucus, epithelial lining fluid, or tissue matrix. Once administered *in vivo*, lipoplexes tend to interact with negatively charged blood components and form large aggregates that could be absorbed onto the surface of circulating red blood cells, trapped in a thick mucus layer, or embolized in microvasculatures, preventing them from reaching the intended target cells in the distal location.[42] Modification of the liposomal surface is a useful way to control the biological properties of liposomes. For example, attachment of specific ligands can enable targeting and accumulation at specific disease sites within the body, and attachment of imaging agents can result in a powerful diagnostic tool. Addition of PEG allows liposomes to circulate longer without being recognized by the body's immune system.

PEG (polyethylene glycol) conjugated cationic liposomes

Anti-cancer drug delivery specifically to cancer cells remains a major challenge. Several approaches, such as liposomes, polymers, polymersome, and micelles carrying anti-cancer drugs, have been utilized for the delivery of drugs to cancer cells, with the expectation of passive targeting through enhanced permeation and retention (EPR) effects. However, lipid-based carriers have been reported to be rapidly cleared from the bloodstream by the reticuloendothelial system (RES). In order to overcome this issue, chemical modification of drug carriers with certain synthetic polymers has been frequently employed in an attempt to increase *in vivo* longevity.[43] The most popular and successful modification is coating with polyethylene glycol (PEG) to achieve "steric stabilization", which hinders the interaction of blood components with their surface and reduces the binding of plasma proteins, toxicity, immunogenicity, and accumulation in the RES.

Poly (ethylene) glycol (PEG) liposomes can be protected from degradation in vivo by surface modifications with polyethylene glycol. PEG has many attractive qualities as a liposomal coating, such as availability in a variety of molecular weights, lack of toxicity, ready excretion by the

kidneys, and ease of application.[44] PEGylated lipoplexes yield increased transfection efficiencies in the presence of serum as compared to liposomal transfection methods lacking such surface attachments.[45] Such liposomes were found to be more stable and displayed longer circulation times in the blood. PEG, being hydrophilic and unable to interact with either DNA or cationic lipids, provides longer circulation times of liposomes in blood circulation by minimizing the binding of blood components and lipoplexes. Unfortunately, inclusion of such bulky PEG lipids into lipoplexes causes dose-dependent inhibition in transfection activity. For this reason a different length of hydrocarbons in PEG-lipid derivatives was used to adjust the time of PEG-lipid association with lipoplexes.[46] The objective of this strategy is to use the PEG as a cover for lipoplexes before they reach the target cells. Once at the target cells, PEG-lipids fall off, revealing highly active lipoplexes. Clinical trials of formulations of PEG-coated liposomal doxorubicin also demonstrated improved pharmacokinetic properties and reduced systemic toxicity.[47-48]

Mukherjee *et al*., 2005 [49] chemically modified haloperidol to conjugate at the distal end of the polyethylene glycol linked phospholipid, which was then incorporated into the cationic liposome known to condense and deliver genes inside cells. The resulting haloperidol-conjugated targeted lipoplex showed at least 10-fold higher ($p < 0.001$) reporter gene expression in MCF-7 cells than control lipoplex. Shroff & Kokkoli, 2012[50] encapsulated doxorubicin inside the liposomes to enhance its therapeutic potential via PEGylation as well as active targeting to the cancer cells. Their results show that PR_b (a fibronectin-mimetic peptide)-functionalized stealth liposomes were able to specifically bind to MDA-MB-231 cells and the binding could be controlled by varying the peptide concentration.

Alternatives to Polyethylene glycol

Some polymers are being used as an alternative to polyethylene glycol with the goal of creating sterically protected lipoplexes. Metselaar *et al*., 2003 [44] reported the use of L-amino-acid-based polymers for lipoplex modification and found an extended circulation time and reduced clearance by macrophages at levels similar to those seen with lipoplexes modified with PEG. These oligopeptides are attractive alternatives to PEG due to advantages such as increased biodegradability and favorable pharmacokinetics when lower concentrations are used per dose.

Zhang *et al*., 2006 [51] described the development of a new strategy for functional siRNA delivery to cells by loading siRNA into liposomes bearing arginine octamer molecules attached to the liposome surface (R8-liposomes). R8 belongs to a large group of so-called cell-penetrating peptides (CPP), which are positively charged and can enter cells when added exogenously.[52]

Role of helper lipid in cationic liposome mediated gene delivery

The mechanism of cationic liposome action is not exactly known. In a majority of reported studies, cationic liposomes function most efficiently when the cationic lipid is mixed with a helper lipid. The most commonly used helper lipid in applications is unsaturated phosphatidylethanolamines (PEs) such as dioleoyl-PE (DOPE).[53-55] Effectiveness of unsaturated PEs, such as DOPE, is believed to rest on their propensity to form nonbilayer structures that are akin to membrane fusion intermediates. This property of helper lipids is thought to facilitate the fusion of cationic liposome in DNA:cationic liposome complexes to cell membranes, thus releasing the DNA into the cytoplasm. Hui *et al*., 1996 [56] studied the role of helper lipids in transfection efficiency, especially in comparing phosphatidylethanolamine (PE) with phosphatidylcholine (PC), which is normally stable as bilayers. Their morphology, uptake route, and kinetics of uptake and transfection were investigated. The function of helper lipid in granule formation on cell surfaces, as found by this work, may be exploited to improve their transfection efficiency. Helper lipid has great impact on the behavior of liposome *in vitro* and *in vivo*. Nie *et al*., 2012 [57] reported anti-cancer effect from charged cholesterol liposome with/without PEGylation for the first time. In order to verify the possible effects from cholesterol charge, surface shielding and chemical nature, two catalogs of liposomes with charged and PEGylated cholesterols were synthesized. It may give deeper understanding on the liposome formulation which is critical for liposome associated drug research and development.

Liposomes for therapeutic applications

From the time when conventional liposomes are digested by phagocytic cells in the body after intravenous management, they are ideal vehicles for targeting drug molecules into these macrophages. Advances in liposome design are leading to new applications for the delivery of new biotechnology products, for example antisense oligonucleotides, cloned genes, and recombinant proteins.[58] Strategic development of drug-loaded nanocarriers tuned to trigger drug release significantly improves the efficacy of drugs and pharmaceuticals. There are several continuing studies with various anti-parasitic liposome formulations in humans. Ligands such as antibodies, peptides, and vitamins (e.g., folic acid), which can bind to upregulated/ overexpressed receptors on tumor tissue, have been investigated as biomarkers for targeted drug delivery.[59] Table 2 lists various drugs that have been encapsulated in liposomes and used for targeting variety of diseases.

Table 2: Therapeutic applications of liposomes

Drug encapsulated in liposome	Type of disease	Ref
Doxorubicin	Cancer	60-61
Amphotericin B	Mycotic infection	62
Insulin	Diabetic mellitus	63
Pentoxyfylline	Asthma	64
Salbutamol	Asthma	65
Levonogesterol	Skin disorder	66
Ibuprofen	Rheumatoid arthritis	66
Idoxiuridine	Rheumatoid arthritis	66
Epaxal	Hepatitis A	33
Inflexal V	Influenza	36
Penicillin	Meningococcal, staphylococcal infection,	66
Methotrexate	Cancer	66
Amphotec	Fungal Infections	30
Diclofenac sodium	Rheumatoid arthritis	67

CONCLUSION

The use of liposomes for gene delivery applications is a huge area and within the frame of a single review paper it is impossible to address all of the pertinent issues. Liposomes promote targeting of particular diseased cells within the disease site. Liposomal drugs exhibit reduced toxicities and retain enhanced efficacy compared with free complements. Long-circulating liposomes are now being investigated in detail and are widely used in biomedical in vitro and in vivo studies; they have also found their way into clinical practice. Cationic lipid-based liposomes are easy to prepare, reasonably cheap and nonimmunogenic. Many of the features of these delivery systems and mechanisms are not sufficiently understood, and so recent studies need to concentrate on structure, function, structure–activity relationships, detailed mechanisms of liposome-mediated gene delivery, and improved efficiency of transfection.

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