Optimization of Non-Viral Gene Therapeutics Using Bilamellar Invaginated Vesicles

Nancy Smyth Templeton^{1,*} and Neil Senzer²

¹Gradalis Inc., 2545 Golden Bear Drive, Suite 110, Carrollton, TX 75006

²Mary Crowley Cancer Research Centers, 1700 Pacific Avenue, Suite 1100, Dallas, TX 75201

Abstract

Bilamellar invaginated vesicles (BIVs) are unique liposomal nanoparticles (NPs) that are highly efficient vehicles for intravenous (iv) delivery of encapsulated therapeutics including plasmid DNA. Systemic administration of therapeutics is required to effectively treat or cure metastatic cancer, certain cardiovascular diseases, and other acquired or inherited diseases. In addition to having extended half-life and stability in circulation, BIVs are nontoxic, nonimmunogenic, biodegradable and can be repeatedly administered without losing potency. Furthermore, BIVs encapsulating therapeutic agents can be modified to specifically enter the disease cells using small molecules that mimic beta turns incorporated on the surface of BIV complexes while focusing biodistribution by bypassing uptake in non-target organs and tissues using reversible masking. These modifications do not alter the unique properties of the BIV delivery system that provide for its robust treatment of disease demonstrated in small and large animal models and in Phase I clinical trials. This review will cover the unique properties of BIVs, including its fusogenic entry into cells and its ability to penetrate tight barriers *in vivo*. Methods to further improve the overall delivery-expression system including further purification of plasmid DNA to eliminate colanic acid from all current commercially produced preparations, and enhanced or prolonged expression provided by plasmid design will also be discussed.

Keywords

Liposomes; Bilamellar invaginated vesicle; Nanoparticle; Plasmid DNA delivery; Gene therapy; Fusogenic entry; Colanic acid; DNA purification

Introduction

Many investigators are focused on the production of effective non-viral gene therapeutics and on creating improved delivery systems that mix desirable features from both viral and non-viral vectors. Use of improved liposome formulations for delivery *in vivo* is valuable for gene therapy and avoids several problems associated with viral delivery. Delivery of nucleic acids using liposomes is promising as a safe and non-immunogenic approach to gene therapy. Furthermore, delivery systems composed of synthetic reagents can be standardized and regulated as drugs rather than as biologics. Cationic lipids have been used for efficient delivery of nucleic acids to cells in tissue culture for several years [1,2]. Much effort has

Copyright: © 2011 Templeton NS, et al.

*Corresponding author: Nancy S. Templeton, Director of Delivery Systems, Gradalis Inc., 2545 Golden Bear Drive, Suite 110, Carrollton, TX 75006-2317, USA. Tel: 214-442-8130; ntempleton@gradalisinc.com.

Citation: Templeton NS, Senzer N (2011) Optimization of Non-Viral Gene Therapeutics Using Bilamellar Invaginated Vesicles. J Genet Syndr Gene Ther S5:002. doi:10.4172/2157-7412.S5-002

also been directed toward developing cationic liposomes for efficient delivery of nucleic acids in animals and in humans [3-12]. Most frequently, the formulations that are best to use for transfection of a broad range of cell types in culture are not optimal for achieving efficacy in small and large animal disease models and in clinical trials.

Non-viral delivery vehicles have numerous advantages over viral vectors that have been used for gene therapy. Following viral delivery *in vivo*, immune responses are generated to expressed viral proteins that, depending on kinetics, can subsequently kill the target cells required to produce the therapeutic gene product. An innate humoral immune response can be produced to certain viral vectors due to previous exposure to the naturally occurring virus. Random integration of some viral vectors into the host chromosome could occur and cause activation of proto-oncogenes resulting in tumor formation, clearance of viral vectors delivered systemically by complement activation can occur. Viral vectors can be inactivated upon re-administration by the induced or secondary humoral immune response, and there is a potential for recombination of a conditionally replicative viral vector that could generate a replication-competent infectious virus. Specific delivery of some of the viral vectors used to target cells can be difficult because two distinct steps in engineering viral envelopes or capsids must be achieved. First, the virus envelope or capsid must be changed to inactivate the natural tropism of the virus to enter off-target cell types. Then sequences must be introduced that allow the new viral vector to bind and internalize through an existing target cell surface receptor. Other disadvantages of viral vectors include the inability to administer certain viral vectors more than once due to elicited neutralizing antibodies, the high costs for producing large amounts of high-titer viral stocks for use in the clinic, and the limited size of the nucleic acid that can be packaged and used for viral gene therapy. Attempts are being made to overcome the immune responses produced by viral vectors after administration in immune competent animals and in humans, such as the use of gutted adenoviral vectors or encapsulation of viral vectors in liposomes [13]. However, complete elimination of all immune responses to viral vectors may be impossible.

Use of liposomes for gene therapy provides several advantages. A major advantage is the lack of immunogenicity after *in vivo* administration including systemic injections. Therefore, the nucleic acid-liposome complexes can be re-administered without harm to the patient and without compromising the efficacy of the non-viral gene therapeutic. Improved formulations of nucleic acid-liposome complexes can also evade complement inactivation after *in vivo* administration. Nucleic acids of unlimited size can be delivered ranging from single nucleotides to large mammalian artificial chromosomes. Furthermore, different types of nucleic acids can be delivered including plasmid DNA, RNA, oligonucleotides, DNA-RNA chimeras, synthetic ribozymes, antisense molecules, RNAi, viral nucleic acids, and others. Certain cationic formulations can also encapsulate and deliver viruses [13], proteins or partial proteins with a low isoelectric point (pI), and mixtures of nucleic acids and proteins of any pI. Creation of non-viral vectors for targeted delivery to specific cell types, organs or tissues is, at least, conceptually simple. Targeted delivery involves elimination of non-specific charge interactions with non-target cells and addition of ligands for binding to target cell surface receptors. Other advantages of non-viral vectors include the low cost and relative ease in producing nucleic acid-liposome complexes in large scale for use in the clinic. In addition, greater safety for patients is provided using non-viral delivery vehicles due to few or no viral sequences present in the nucleic acids used for delivery, thereby precluding generation of an infectious virus. The disadvantage of non-viral delivery systems has been the low levels of delivery and gene expression produced by “first-generation” complexes. However, recent advances using BIVs have dramatically improved transfection efficiencies and efficacy of liposomal vectors [14-20].

Cationic liposome-nucleic acid complexes can be administered via numerous delivery routes *in vivo*. Routes of delivery include direct injection (e.g. intratumoral), intravenous, intraperitoneal, intraarterial, intrasplenic, mucosal (nasal, vaginal, rectal), intramuscular, subcutaneous, transdermal, intradermal, subretinal, intratracheal, intracranial, and others. Much interest has focused on intravenous administration because many investigators believe that this easily accessible and reusable systemic route of delivery is the “holy grail” for the treatment or cure of cancer, cardiovascular and other inherited or acquired diseases. Particularly for the treatment of metastatic cancer, therapeutics must reach not only the primary tumor but also the distant metastases.

Optimization of cationic liposomal complexes for *in vivo* applications and therapeutics is complex involving many distinct components including nucleic acid purification, plasmid design, formulation of the delivery vehicle, administration route and schedule, dosing, detection of gene expression, and others. Often we make the analogy of liposome optimization to a functional car. Of course the engine of the car, analogous to the liposome delivery vehicle, is extremely important. However, if the car does not have wheels, adequate tires, etc., the motorist will not be able to drive the vehicle to its destination, and without a map or GPS he/she may not arrive to the right destination. This review will focus on optimization of these distinct components for use in a variety of *in vivo* applications. Optimizing all components of the delivery system will allow broad use of NP complexes to treat or cure human diseases or disorders.

Optimization of cationic liposome formulations for use *in vivo*

Much research has been directed toward the synthesis of new cationic lipids. Some new formulations enable more efficient transfection of cells in culture. However, their efficiency measured *in vitro* did not correlate with their ability to deliver DNA after administration in animals. Functional properties defined *in vitro* do not assess the stability of the complexes in plasma or their pharmacokinetics and biodistribution, all of which are essential for optimal activity *in vivo*. Colloidal properties of the complexes, in addition to the physicochemical properties of their component lipids, also determine these parameters. In particular, in addition to efficient transfection of target cells, nucleic acid-liposome complexes must be able to traverse tight barriers *in vivo* and penetrate throughout the target tissue to produce efficacy for the treatment of disease, i.e. countercurrent to increased intratumoral pressure gradients for the treatment of cancer. These are not issues for achieving efficient transfection of cells in culture with the exception of polarized tissue culture cells. Therefore, we are not surprised that optimized liposomal delivery vehicles for use *in vivo* may be different than those used for efficient delivery to some cells in culture.

In summary, *in vivo* nucleic acid-liposome complexes that produce efficacy in animal models of disease have extended half-life in the circulation, are stable in serum, have broad biodistribution that can be focused, efficiently encapsulate various sizes of nucleic acids, are targetable to specific organs and cell types, penetrate across tight barriers in several organs, penetrate evenly throughout the target tissue, are optimized for nucleic acid:lipid ratio and colloidal suspension *in vivo*, can be size fractionated to produce a homogenous population of complexes prior to injection, and can be repeatedly administered. Recently, we demonstrated efficacy of a robust liposomal delivery system in small and large animal models for lung [15,16], breast [18], head and neck, and pancreatic cancers [17,21,22], and for Hepatitis B and C [23]. Based on efficacy in these animal studies, this liposomal delivery system has been used successfully in phase I clinical trials to treat end-stage non-small cell lung carcinoma patients who have failed to respond to chemotherapy [24] and hereditary inclusion body myopathy [25,26]. The non-small cell lung carcinoma patients have prolonged life spans and have demonstrated objective responses including tumor regression. Efficacy was also demonstrated for the single patient trials for hereditary inclusion body

myopathy. The BIV delivery system will also be used in upcoming clinical trials to treat other types of cancer including pancreatic, breast, head and neck cancers, and others. Our studies have demonstrated broad efficacy in the use of liposomes to treat disease and have dispelled several myths that exist concerning the use of liposomal systems.

Liposome morphology and effects on gene delivery and expression

Efficient *in vivo* nucleic acid-liposome complexes have unique features including their morphology, mechanisms for crossing the cell membrane and entry into the nucleus, ability to be targeted for delivery to specific cell surface receptors, and ability to penetrate across tight barriers and throughout target tissues. Liposomes have different morphologies based upon their composition and the formulation method. Furthermore, the morphology of complexes can contribute to their ability to deliver nucleic acids *in vivo*. Formulations frequently used for the delivery of nucleic acids are lamellar structures including small unilamellar vesicles (SUVs), multilamellar vesicles (MLVs), or the bilamellar invaginated vesicles (BIVs) recently developed in our laboratory (Figure 1). Several investigators have developed liposomal delivery systems using hexagonal structures, however, they have demonstrated efficiency primarily for the transfection of some cell types in culture and not for *in vivo* delivery. SUVs condense nucleic acids on the surface and form “spaghetti and meatballs” structures [27]. DNA-liposome complexes made using SUVs produce little or no gene expression upon systemic delivery, although these complexes transfect numerous cell types efficiently *in vitro* [1,2]. Furthermore, SUV liposome-DNA complexes cannot be targeted efficiently. SUV liposome-DNA complexes also have a short half-life within the circulation, generally about 5 to 10 minutes. Polyethylene glycol (PEG) has been added to liposome formulations to extend their half-life [28-30], however, PEGylation creates other problems that have not as yet been resolved. PEG seems to hinder delivery of cationic liposomes into cells due to its sterically hindering ionic interactions, and it interferes with optimal condensation of nucleic acids onto the cationic delivery vehicle. Furthermore, the resultant extremely long half-life in the circulation, e.g. up to several days, has caused problems for patients as illustrated by the increased percentage of injected dose of the PEGylated liposomal formulation doxil that encapsulates the cytotoxic agent, doxorubicin, which accumulates in the skin, hands, and feet resulting in mucositis and Hand and Foot Syndrome [31,32] that cause extreme discomfort to the patient. Attempts to add ligands to doxil for delivery to specific cell surface receptors has not resulted in much cell-specific delivery, and an increased percentage of the injected targeted formulation still accumulates in the skin, hands, and feet. Addition of PEG into formulations developed in our laboratory also caused steric hindrance in the bilamellar invaginated structures that hindered DNA encapsulation, and gene expression was substantially diminished. Recent efforts to use cleavable PEG are unimpressive and have not solved these problems [33-40]. The vast majority of the injected PEGylated complexes bypass the target cell, including those using cleavable PEG.

Some investigators have loaded nucleic acids into SUVs using a variety of methods; however, the bulk of the DNA does not load or stay within the liposomes. Furthermore, most of the processes used for loading nucleic acids within liposomes are extremely time-consuming and not cost effective. Therefore, SUVs are not the ideal liposomes for creating non-viral vehicles for targeted delivery.

Complexes made using MLVs appear as “Swiss rolls” when viewing cross-sections by cryo-electron microscopy [41]. These complexes can become too large for systemic administration or deliver nucleic acids inefficiently into cells due to inability to “unravel” at the cell surface. Addition of ligands onto MLV liposome-DNA complexes further aggravates these problems. Therefore, MLVs are not useful for the development of targeted delivery of nucleic acids.

Using a formulation developed in our laboratory, nucleic acids are efficiently encapsulated between two bilamellar invaginated vesicles, BIVs [14]. We created these unique structures using 1,2-bis(oleoyloxy)-3-(trimethylammino)propane (DOTAP) and synthetic cholesterol (Chol) and a novel formulation procedure. This procedure is different because it includes a brief, low frequency sonication followed by manual extrusion through filters of decreasing pore size. The 0.1 and 0.2 μm filters used are made of aluminum oxide and not polycarbonate that is typically used by other protocols. Aluminum oxide membranes contain more pores per surface area that are evenly spaced and sized, and have straight channels. During the manual extrusion process the liposomes are passed through each of four different sized filters only once. This process produces 88% invaginated liposomes. Use of high frequency sonication and/or mechanical extrusion produces only SUVs.

BIVs condense unusually large amounts of nucleic acids of any size (Figure 2) as well as viruses (Figure 3). Furthermore, addition of other DNA condensing agents including polymers is not necessary. For example, condensation of plasmid DNA onto polymers prior to encapsulation in the BIVs did not increase condensation or subsequent gene expression after transfection *in vitro* or *in vivo*. Encapsulation of nucleic acids by these BIVs alone is spontaneous and immediate, and therefore, cost effective requiring only one step of simple mixing. The extruded BIV DOTAP: Chol-nucleic acid complexes are also large enough so that they are not cleared rapidly by Kupffer cells in the liver and yet extravasate across tight barriers, including the endothelial cell barrier of the lungs in a normal mouse, and diffuse through target organs efficiently [15]. Our work demonstrating efficacy for treatment of non-small cell lung cancer [15] showed that only BIV DOTAP:Chol-p53 DNA liposome complexes produced efficacy; whereas SUV DOTAP:Chol-p53 DNA liposome complexes produced no efficacy. Therefore, the choice of lipids alone is not sufficient for optimal DNA delivery, and the morphology of the complexes is essential.

Optimal lipids and liposome morphology: effects on gene delivery and expression

Choosing the best cationic lipids and neutral lipids are also essential for producing the optimal *in vivo* formulation. For example, using our novel manual extrusion procedure does not produce BIVs using the cationic lipid dimethyldioctadecylammonium bromide (DDAB). Furthermore, DOTAP is biodegradable, whereas DDAB is not biodegradable. Use of biodegradable lipids is preferred for use in humans. Furthermore, only DOTAP and not DDAB containing liposomes produced highly efficient gene expression *in vivo* [14]. DDAB did not produce BIVs and was unable to encapsulate nucleic acids. Apparently, DDAB and DOTAP containing SUVs produce similar efficiency of gene delivery *in vivo*; however, these SUVs are not as efficient as BIV DOTAP:Chol [14]. In addition, use of L- α dioleoyl phosphatidylethanolamine (DOPE) as a neutral lipid creates liposomes that cannot wrap or encapsulate nucleic acids. Several investigators have reported efficient transfection of cells in culture using DOPE in liposomal formulations. However, our data showed that formulations consisting of DOPE were not efficient for producing gene expression *in vivo* [14].

Investigators must also consider the source and lot variability of certain lipids purchased from companies. For example, different lots of natural cholesterol from the same vendor can vary dramatically and will affect the formulation of liposomes. We use synthetic cholesterol instead of natural cholesterol that is purified from the wool of sheep. Synthetic cholesterol is required by the Food and Drug Administration for use in producing therapeutics for injection into humans.

Our BIV formulations are also stable for a few years as liquid suspensions. Freeze-dried formulations can also be made that are stable indefinitely even at room temperature.

Stability of liposomes and liposomal complexes is also essential particularly for the commercial development of human therapeutics.

Liposome encapsulation, flexibility and optimal colloidal suspensions

A common belief is that artificial vehicles must be 100 nm or smaller to be effective for systemic delivery. However, this belief is most likely true only for large, inflexible delivery vehicles. Blood cells are several microns (up to 7000 nm) in size, and yet have no difficulty circulating in the blood including through the smallest capillaries. However, sickle cell blood cells, that are rigid, do have problems in the circulation. Therefore, we believe that flexibility is a more important issue than small size. In fact, BIV DNA-liposome complexes in the size range of 200 to 450 nm produced the highest levels of gene expression in all tissues after iv injection [14]. Delivery vehicles, including non-viral vectors and viruses, that are not PEGylated and are smaller than 200 nm are cleared quickly by the Kupffer cells in the liver. Therefore, increased size of liposomal complexes could extend their circulation time particularly when combined with injection of high colloidal suspensions. BIVs are able to encapsulate nucleic acids and viruses apparently due to the presence of cholesterol in the bilayer (Figure 4). Formulations including DOPE instead of cholesterol could not assemble nucleic acids by a “wrapping type” of mechanism (Figure 5), and produced little gene expression in the lungs and no expression in other tissues after intravenous injections. Because the extruded DOTAP: Chol BIV complexes are flexible and not rigid, are stable in high concentrations of serum, and have extended half-life, they do not have difficulty circulating efficiently in the bloodstream.

We believe that colloidal properties of nucleic acid-liposome complexes also determine the levels of gene expression produced after *in vivo* delivery [14,42] These properties include the DNA: lipid ratio that determines the overall charge density of the complexes and the colloidal suspension that is monitored by its turbidity. Complex size and shape, lipid composition and formulation, and encapsulation efficiency of nucleic acids by the liposomes also contribute to the colloidal properties of the complexes. The colloidal properties affect serum stability, protection from nuclease degradation, blood circulation time, and biodistribution of the complexes.

Our *in vivo* transfection data showed that an adequate amount of colloids in suspension was required to produce efficient gene expression in all tissues examined [14]. The colloidal suspension is assessed by measurement of adsorbance at 400 nm using a spectrophotometer optimized to measure turbidity. Our data showed that transfection efficiency in all tissues correlated with OD400 of the complexes measured prior to intravenous injection.

Overall charge of complexes and entry into the cell

In addition, our delivery system is efficient because the complexes deliver DNA into cells by fusion with the cell membrane and avoid the endocytic pathway (Figure 6). Cells are negatively charged on the surface, and specific cell types vary in their density of negative charge. These differences in charge density can influence the ability of cells to be transfected. Cationic complexes have non-specific ionic charge interactions with cell surfaces. Efficient transfection of cells by cationic complexes is, in part, contributed by adequate charge interactions. In addition, recent publications report that certain viruses have a partial positive charge around key subunits of viral proteins on the virus surface responsible for binding to and internalization through target cell surface receptors. It appears that this partial positive charge is required for virus entry into the cell [43-48]. Thus, maintenance of adequate positive charge on the surface of targeted liposome complexes is essential for optimal delivery into the cell. Different formulations of liposomes interact with cell surfaces via a variety of mechanisms. Two major pathways for interaction are by

endocytosis or by direct fusion with the cell membrane [41,49-54]. Preliminary data suggest that nucleic acids delivered *in vitro* and *in vivo* using complexes developed in our lab enter the cell by direct fusion (Figure 6). Apparently, the bulk of the nucleic acids do not enter endosomes, and therefore, the nucleic acids enter the nucleus rapidly. Fusogenic cell transfection produced orders of magnitude increased levels of gene expression and far greater numbers of cells transfected versus cells transfected through the endocytic pathway.

Targeted delivery and reversible masking

There are two components to optimal targeted delivery: focused biodistribution and uptake exclusively in the target cell. We believe that adequate positive charge exposure on the surface of complexes is essential for both target-selective biodistribution and to drive cell entry by direct fusion. Therefore, we create ligand-facilitated targeted delivery of our complexes *in vivo* without the use of PEG. These ligand-coated complexes are constructed to re-expose the overall positive charge of the complexes as they approach the target cells. Through ionic interactions or covalent attachments, we have added monoclonal antibodies, Fab fragments, proteins, partial proteins, peptides, peptide mimetics, small molecules and drugs to the surface of our complexes after mixing. However, we believe that the covalent attachment of small molecules that mimic protein-protein interactions are best to use for specific target cell uptake in order to prevent immune responses upon repeated administration. These ligands efficiently bind to the target cell surface receptor yet retain fusogenicity. Using novel methods for addition of ligands to the complexes for targeted delivery results in further increased gene expression in the target cells after transfection. We also use reversible masking to avoid nonspecific uptake [20] (Templeton, N.S. US Patent No. 7,037,520 B2 issued May 2, 2006). Briefly, we add the small neutral lipid, *n*-dodecyl- β -D-maltopyranoside, approximately 511 MW, just prior to iv injections. Therefore, we designed targeted liposomal delivery systems that retain predominant entry into cells by direct fusion versus the endocytic pathway. Figure 7 shows our optimized strategy to achieve targeted delivery, deshielding, fusion with the cell membrane, entry of nucleic acids into the cell and into the nucleus, and production of gene expression of a cDNA cloned in a plasmid.

We have developed a multi-disciplinary approach combining molecular biology, delivery technology, combinatorial chemistry, and reversible masking to create improved systemic, targeted delivery of plasmid DNA while avoiding non-specific uptake *in vivo*. We applied this technology to efficiently target delivery to a human tumor-microenvironment model [20]. We achieved efficient, targeted delivery by attachment of specific targeting ligands to the surface of our BIV complexes in conjunction with reversible masking to bypass nonspecific tissues and organs. We identified ligands that target a human tumor-microenvironment created *in vitro* by co-culturing primary human endothelial cells with human lung or pancreatic cancer cells. The model was confirmed by increased expression of tumor endothelial markers including CD31 and VEGF-A, and prolonged survival of endothelial capillary-like structures. The co-cultures were used for high-throughput screening of a specialized small-molecule peptidomimetic library to identify ligands specific for human tumor-associated endothelial cells *in vitro*. We identified small molecules that enhanced the transfection efficiency of tumor-associated endothelial cells, but not normal human endothelial cells or cancer cells. IV administration of our targeted, reversibly masked complexes into mice bearing human pancreatic tumor and endothelial cells specifically increased transfection to this tumor microenvironment about 200-fold. Efficacy studies using our optimized targeted delivery of a plasmid encoding thrombospondin-1 eliminated tumors completely after five intravenous injections administered once weekly. We plan to use our targeted, reversibly masked delivery system in the clinic to treat metastatic cancer.

Serum stability of optimized nucleic acid-liposome complexes for use *in vivo*

Serum stability of cationic complexes is complicated and cannot be assessed by simply performing studies at a random concentration of serum. Figure 8 shows results from serum stability studies of DNA-liposome complexes that have been optimized in our laboratory for systemic delivery. Serum stability of these complexes was studied at 37°C out to 24 hours at concentrations of serum ranging from 0 to 100%. Two different serum stability assays were performed. The first assay measured the OD400 of BIV DOTAP: Chol-DNA liposome complexes added into tubes containing a different concentration of serum in each tube, ranging from 0 to 100%. The tubes were incubated at 37°C and small aliquots from each tube were removed at various time points out to 24 hours. The OD400 of each aliquot was measured on a spectrophotometer calibrated to accurately measure turbidity. Previous work in our laboratory demonstrated that the OD400 predicted both the stability of the complexes and the transfection efficiency results obtained for multiple organs after intravenous injections [14, 42]. Percent stability for this assay is defined as the transfection efficiency that is obtained at a particular OD400 of the complexes used for intravenous injections. Therefore, this assay is rigorous because slight declines in OD400 of these complexes correlate with loss of transfection *in vivo*. Declines in the OD400 also measure precipitation of the complexes.

A second assay was performed to support the results obtained from the OD400 measurements described above. A different concentration of serum, ranging from 0 to 100%, was placed into each well of a 96-well micro-titer dish. BIV DOTAP:Chol-DNA liposome complexes were added to the serum in the wells, and the plate was incubated at 37°C. The plate was removed at various time points out to 24 hours and complexes in the wells were observed under the microscope. Precipitation of complexes in the wells was assessed. 100% stability was set at no precipitation observed. Results from this assay were compared to those obtained in the first assay. 100% stability of complexes was set at no decline of OD400 in assay #1 and no observed precipitation in assay #2 at each % serum concentration, and the results were plotted (Figure 8).

The results showed serum stability at the highest concentrations of serum, about 70 to 100%, that are physiological concentrations of serum found in the bloodstream. In addition, these complexes were also stable in no or low concentrations of serum. However, the complexes were unstable at 10 to 50% serum, perhaps due to salt bridging. Therefore, *in vitro* optimization of serum stability for formulations of cationic complexes must be performed at high serum concentrations to predict efficacy *in vivo*. Results from the Barenholz laboratory also confirmed the serum stability of DOTAP: Chol liposomes and DOTAP: Chol complexes *in vivo* [55].

Optimized half-life in the circulation

As stated above, the extruded BIV DOTAP: Chol-nucleic acid complexes are large enough so that they are not cleared rapidly by Kupffer cells in the liver and yet extravasate across tight barriers and diffuse through the target organ efficiently. Further addition of ligands to the surface of extruded BIV DOTAP: Chol-nucleic acid complexes does not significantly increase the mean particle size. Extravasation and penetration through the target organ and gene expression produced after transfection are not diminished. We have demonstrated delivery across the tight posterior blood retinal barrier in the eyes of adult mice [56], across tight layers of smooth muscle cells in the arteries of pigs after balloon angioplasty [56], across the tight endothelial cell barrier of normal mice [15,56], and across the interstitial pressure gradient of large solid tumors [15,56]. These BIVs are positively charged and deliver nucleic acids efficiently into cells *in vitro* and *in vivo*. Because extruded BIV DOTAP: Chol-nucleic acid complexes with or without ligands have a five-hour half-life in

the circulation, these complexes do not accumulate in the skin, hands or feet. Extended half-life in the circulation is provided primarily by the formulation, preparation method, injection of optimal colloidal suspensions, and optimal nucleic acid:lipid ratio used for mixing complexes, serum stability, and size (200 to 450 nm). Therefore, these BIVs are ideal for use in the development of effective, targeted non-viral delivery systems that require encapsulation of nucleic acids.

Broad biodistribution of optimized liposome formulations

Our “generic” BIV nucleic acid-liposome formulation transfects many organs and tissues efficiently after intravenous injection [14] and has demonstrated efficacy in animal models for lung cancer [15,16], breast cancer [18], pancreatic cancer [17,21,22], Hepatitis B and C [23], and cardiovascular diseases [56]. Many additional publications have validated the efficacy of DOTAP: Cholesterol liposomes for use in treating several different types of cancer. Our BIVs have been broadly used world wide to effectively treat many cancers. Therefore, optimization of the morphology of the complexes, the lipids used, flexibility of the liposomes and complexes, colloidal suspension, overall charge, serum stability, and half-life in circulation allows for efficient delivery and gene expression in many organs and tissues other than the lung. Apparently, these extruded DOTAP: Chol BIV nucleic acid-liposome complexes can overcome the tendency to be adsorbed only by the endothelial cells lining the circulation surrounding the lungs described by other investigators [57]. However, as discussed above and below, we can further direct delivery to specific target tissues or cells by our targeted delivery strategies in combination with reversible masking used to bypass non-specific transfection.

Efficient dissemination throughout target tissues and migration across tight barriers

A primary goal for efficient *in vivo* delivery is to achieve extravasation into and penetration throughout the target organ/tissue ideally by minimally-invasive systemic administration. Without these events therapeutic efficacy is highly compromised for any treatment including gene and drug therapies. Achieving this goal is difficult due to the many tight barriers that exist in animals and people. Furthermore, many of these barriers become tighter in the transition from neonates to becoming adults. Penetration throughout an entire tumor is further hindered due to the increased interstitial pressure within most tumors [58-60]. We believe that non-viral systems can play a pivotal role in achieving target organ extravasation and penetration needed to treat or cure certain diseases. Our preliminary studies have shown that extruded BIV DOTAP: Chol nucleic acid:liposome complexes can extravasate across tight barriers and penetrate evenly throughout entire target organs; whereas viral vectors cannot cross identical barriers. As stated above, these barriers include the endothelial cell barrier in a normal mouse [15,56], the posterior blood retinal barrier in adult mouse eyes [56], complete and homogeneous diffusion throughout large tumors [15,56], and penetration through several tight layers of smooth muscle cells in the arteries of pigs [56]. Diffusion throughout large tumors was measured by expression of β -galactosidase or the pro-apoptotic gene p53 in about half of the p53-null tumor cells after a single injection of BIV DOTAP: Chol-DNA liposome complexes into the center of a tumor. Transfected cells were evenly spread throughout the tumors. Tumors injected with complexes encapsulating plasmid DNA encoding p53 showed apoptosis in almost all of the tumor cells by TUNEL staining. Tumor cells expressing p53 mediate a bystander effect on neighboring cells perhaps due to up-regulation by Fas ligand that causes non-transfected tumor cells to undergo apoptosis.

Optimization of plasmids for gene expression

Delivery of DNA may not correlate with subsequent gene expression [19,61]. Investigators may focus solely on the delivery formulation as the source of poor gene expression. In many cases, however, the delivery of DNA into the nucleus of a particular cell type may be

efficient, although little or no gene expression is achieved. The causes of poor gene expression can be numerous. The following issues should be considered independent of the delivery formulation: sub-optimal promoter-enhancers in the plasmid, absence of the appropriately modified histones associated with the plasmid in the nucleus, poor preparation of plasmid DNA, and insensitive detection of gene expression. For example, many investigators have shown that performing *in vitro* transfection in the presence of histone deacetylase inhibitors dramatically increased gene expression [61].

Plasmid expression cassettes typically have not been optimized for animal studies. For example, many plasmids lack a full-length CMV promoter-enhancer. Over one hundred variations of the CMV promoter-enhancer exist, and some variations produce greatly reduced or no gene expression in certain cell types [19]. Even commercially available plasmids contain sub-optimal CMV promoters-enhancers, although these plasmids are advertised for use in animals. Furthermore, upon checking the company data for these plasmids, one discovers that these plasmids have never been tested in animals and have been tested in only one or two cultured cell lines. Conversely, plasmids that have been optimized for overall efficiency in animals may not be best for transfection of certain cell types *in vitro* or *in vivo*. For example, the expression levels achieved using optimal CMV promoters-enhancers can vary several orders of magnitude among different certain cell types. In addition, one cannot assume that a CMV promoter that expresses well within the context of a viral vector, such as adenovirus, will function as well in a plasmid-based transfection system for the same cell context. Virus proteins produced by the viral vector are required for producing high levels of mRNA by the CMV promoter in specific cell nuclei.

Ideally, investigators design custom promoter-enhancer chimeras that produce the highest levels of gene expression in their target cells of interest. Recently, we designed a systematic approach for customizing plasmids used for breast cancer gene therapy using expression profiling [19]. Furthermore, more sophisticated design may be needed to promote highly efficient transcription elongation [62]. Gene therapy clinical trials for cancer frequently produce inconsistent results. We believe that some of this variability could result from differences in transcriptional regulation that limit expression of therapeutic genes in specific cancers. Our systemic liposomal delivery of a non-viral plasmid DNA showed efficacy in animal models for several cancers. However, we observed large differences in the levels of gene expression from a CMV promoter-enhancer between lung and breast cancers [19]. To optimize gene expression in breast cancer cells *in vitro* and *in vivo*, we created a new promoter-enhancer chimera to regulate gene expression. Serial analyses of gene expression (SAGE) data from a panel of breast carcinomas and normal breast cells predicted promoters that are highly active in breast cancers, for example the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter. Furthermore, GAPDH is up-regulated by hypoxia, which is common in tumors. We added the GAPDH promoter, including the hypoxia enhancer sequences, to our *in vivo* gene expression plasmid. The novel CMV-GAPDH promoter-enhancer showed up to 70-fold increased gene expression in breast tumors compared to the optimized CMV promoter-enhancer alone. No significant increase in gene expression was observed in other tissues. These data demonstrate tissue specific effects on gene expression after non-viral delivery and suggest that gene delivery systems may require plasmid modifications for optimal treatment of different tumor types. Furthermore, expression profiling can facilitate the design of optimal expression plasmids for use in specific cancers.

Several reviews have stated that non-viral systems are intrinsically inefficient compared to viral systems. However, as discussed above, one must separate issues of the delivery vehicle versus the plasmid that is delivered. Case in point, we have shown that our BIV liposomes optimized for systemic delivery could out-compete delivery using a lentivirus. For example,

we have compared SIVmac239, a highly non-infectious virus, with non-viral delivery of SIVmac239 DNA complexed to BIVs in adult rhesus macaques after injection into the saphenous vein of the leg [56]. Our data showed that the monkeys injected with SIV DNA encapsulated in DOTAP: Chol BIVs were infected four days post-injection, and high levels of infection were produced in these monkeys at 14 days post-injection. Furthermore, higher levels of SIV RNA in the blood were produced using our BIV liposomes for delivery versus using the SIV virus. CD4⁺ T cell counts were measured before and after injections. CD4 levels dropped in all monkeys to the lowest levels ever detected in the macaques in any experiment by 28 days post-injection, the first time point at which these counts were measured post-injection. All monkeys had clinical SIV infections and lost significant weight by day 28. These results were surprising because SIVmac239 is not highly infectious, and monkeys become sick with SIV infection only after several months or years post-injection with SIVmac239 virus. Therefore, we were able to induce SIV infection faster using our non-viral delivery of SIV plasmid DNA. In this case, we delivered a replication competent plasmid so that gene expression increased over time post-transfection. Our delivery system was highly efficient and exceeded that of the lentivirus. The critical feature in this non-viral experiment was the plasmid DNA that was delivered. Our BIV targeted delivery system has also surpassed the lentivirus system for transfection of lymphocytes in culture and circulating lymphocytes *in vivo* [63].

Plasmids can be engineered to provide for tissue specific or long-term gene expression, replication, or integration. Persistence elements, such as the inverted terminal repeats from adenovirus or adeno-associated virus, have been added to plasmids to prolong gene expression *in vitro* and *in vivo* [9,11,64]. Apparently, these elements bind to the nuclear matrix thereby retaining the plasmid in cell nuclei. For regulated gene expression, many different inducible promoters are used that promote expression only in the presence of a positive regulator or in the absence of a negative regulator. Tissue specific promoters have been used for the production of gene expression exclusively in the target cells. As discussed in the previous paragraph, replication competent plasmids or plasmids containing sequences for autonomous replication can be included that provide prolonged gene expression. Other plasmid-based strategies produce site-specific integration or homologous recombination within the host cell genome [65,66]. Integration of a cDNA into a specific “silent site” in the genome could provide long-term gene expression without disruption of normal cellular functions. Homologous recombination could correct genetic mutations upon integration of wild-type sequences that replace mutations in the genome. Much work is being conducted in the use of transposons, including the *Sleeping Beauty* transposon system for insertion of non-viral sequences into host chromosomes [66].

Optimization of plasmid DNA preparations

The transfection efficiency of plasmid DNA is dependent on the preparation protocol and training of the person preparing the DNA. For example, we performed a blinded study asking three people to make DNA preparations of the same plasmid from the same box of a Qiagen Endo-Free Plasmid Preparation kit. One person then mixed all of the DNA-liposome complexes on the same morning using a single vial of liposomes. One person performed all tail vein injections, harvesting of tissues, preparation of extracts from tissues, and reporter gene assays on the tissue extracts. *In vivo* gene expression differed 30-fold among these three plasmid DNA preparations.

One source for this variability is that optimized methods to detect and remove contaminants from plasmid DNA preparations have not been available. We have identified large amounts of contaminants, particularly colanic acid, that exist in laboratory and clinical grade preparations of plasmid DNA [67]. Colanic acid and other non-endotoxin associated polysaccharides co-purify with DNA by anion exchange chromatography and by cesium

chloride density gradient centrifugation. Endotoxin removal protocols do not remove these contaminants, and standard analytical methods including HPLC cannot detect these contaminants. Therefore, we developed six methods for the detection of these contaminants in plasmid DNA preparations [67]. We are making clinical grade (GMP) DNA that does not contain these contaminants. To provide the greatest efficacy and levels of safety, these contaminants, particularly colanic acid, must be assessed and removed from plasmid DNA preparations. Polysaccharides are known to inhibit both DNA and RNA polymerase activities [67]. Therefore, gene expression post-transfection can be increased by orders of magnitude if these contaminants are removed from DNA preparations. The presence of these contaminants in DNA results in toxicity, including death at high doses, when DNA-liposome complexes are injected intravenously. Our group and other investigators have shown that intravenous injections of high doses of improved liposomes alone cause no adverse effects in small and large animals, and removal of colanic acid eliminates the toxicity observed for DNA-liposome complexes.

Removing all or the majority of CpG sequences from plasmids is reported to reduce toxicity after intravenous injections of cationic liposomes complexed to these plasmids [68]. However, the beneficial effects were only observed using low doses containing up to 16.5 ug of DNA per injection into each mouse. Achieving efficacy for cancer metastases, particularly in mice bearing aggressive tumors, typically requires injecting higher doses in the range of 50 to 150 ug of DNA per mouse. Therefore, removal of CpG sequences from plasmid based gene therapy vectors will not be useful for applications that require high doses. CpG removal did not reduce toxicity after iv injections of complexes with higher doses of plasmid DNA [68]. Therefore, we believe that the failure to remove specific contaminants in current plasmid DNA preparations, particularly colanic acid, is the major block to the safe intravenous injection of high doses of DNA-liposome complexes.

Detection of gene expression

Thought should also be given to choosing the most sensitive detection method for every application of non-viral delivery rather than using the method that seems most simple. For example, detection of β -galactosidase expression is far more sensitive than that for the green fluorescent protein (GFP). Specifically, five hundred molecules of β -galactosidase (β -gal) per cell are required for detection using X-gal staining. Whereas, about 50,000 molecules of GFP per cell are required for direct detection. Furthermore, detection of GFP may be impossible if the fluorescence background of the target cell or tissue is too high. Detection of chloramphenicol acetyltransferase (CAT) is extremely sensitive with no background detected in untransfected mammalian cells *in vitro* or *in vivo*. Often, assays for CAT expression can provide more useful information than using β -gal or GFP as reporter genes. PCR detection of plasmid DNA or mRNA is also flawed with high background, and the data gathered from transfection can often be misleading.

Luciferase can be detected with high sensitivity by luminescence assays of cell or tissue extracts post-transfection. However, luciferase expression may not predict the therapeutic potential of a non-viral delivery system. For example, if several hundred or thousand molecules per cell of a therapeutic gene are required to produce efficacy for a certain disease, then production of only few molecules will not be adequate. Thus luciferase expression for a given target cell and specific non-viral delivery system must be carefully quantified to avoid being misled about its therapeutic potential.

Noninvasive detection of luciferase expression *in vivo* is not as sensitive as *in vitro* luminescence assays. Some of our colleagues have tried cooled charge coupled device (CCD) camera imaging on live mice after intravenous injection of other cationic liposomes complexed to plasmid DNA encoding luciferase, and they were not able to detect any

transfection even though these liposomal delivery systems had been used to detect luciferase by luminescence assays of organ extracts. However, they were able to detect luciferase expression by CCD imaging after intravenous injections of BIV DOTAP: Chol-luciferase DNA-liposome complexes [69].

Because the luciferase protein is short-lived, maximal expression was detected at 5 hours post-transfection; whereas, detection of HSV-TK gene expression using microPET imaging in the same mice was highest at 24 hours post-transfection. In contrast to luciferase, the CAT protein accumulates over time, and therefore, the investigator is not restricted to a narrow time frame for detecting gene expression. Furthermore, detection of CAT seems to be more sensitive than CCD imaging of luciferase following intravenous injections of DNA-liposome complexes. However, the animals must be sacrificed in order to perform CAT assays on tissue or organ extracts. We and several investigators are interested in PET, PET/CT, and MRI imaging; however, these systems require further research and development in order to produce sensitive and accurate tracking of plasmid DNA *in vivo*. In summary, further work is still needed to develop *in vivo* detection systems that have high sensitivity and low background.

Optimization of dose and frequency of administration

To maximize efficacy for treatment of certain diseases and to create robust vaccines, injections or administrations of the non-viral gene therapeutic, *etc.* via different routes may be required. For particular treatments, one should not assume that one delivery route is superior to others without performing the appropriate animal experiments. In addition, people with the appropriate expertise should perform the injections and administrations. In our experience, only a minority of people who claim expertise in performing tail vein injections can actually perform intravenous injections.

The optimal dose should be determined for each therapeutic gene or other nucleic acid that is administered. The investigator should not assume that the highest tolerable dose is optimal for producing maximal efficacy. The optimal administration schedule should also be determined for each therapeutic gene or other nucleic acid. For example, some investigators have simply used the same administration schedule that they used for chemotherapeutics. The investigator should perform *in vivo* experiments to determine when gene expression and/or efficacy drop significantly. Most likely, re-administration of the non-viral gene therapeutic is not necessary until this drop occurs. For example, we found that iv injections of BIV complexes was optimal using weekly injections for a total of five injections to eliminate pancreatic tumors [20]; whereas three injections administered once every two weeks over six weeks was slightly less effective. Duration of therapeutic efficacy will vary with the half-life of the protein produced. Therefore, if a therapeutic protein has a longer half-life, then the gene therapy could be administered less frequently.

Summary

Some hurdles remain to be overcome before the broad application of non-viral delivery can be achieved; however, we are confident that we will successfully accomplish these remaining challenges. Furthermore, we predict that eventually the majority of gene therapies will utilize synthetic delivery vehicles that can be standardized and regulated as drugs rather than biologics. We will continue to emulate those molecular mechanisms of viral delivery that produce efficient delivery to cells into artificial systems. Therefore, the synthetic delivery systems, including liposomal delivery vehicles, will be further engineered to mimic the most beneficial aspects of the viral delivery systems while circumventing their limitations. We will also further optimize the numerous benefits of the liposomal delivery systems discussed in this chapter.

Acknowledgments

This work was supported by NCI/NIH HHSN261201100055C and NCI/NIH HHSN261201000108C.

Abbreviations

BIV	bilamellar invaginated vesicle
NP	nanoparticle
iv	Intravenous
CA	colanic acid
CAE	colanic acid degrading enzyme

References

1. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, et al. Lipofection: a highly efficient lipid-mediated DNA transfection procedure. *Proc Natl Acad Sci U S A*. 1987; 84:7413–7417. [PubMed: 2823261]
2. Felgner JH, Kumar R, Sridhar CN, Wheeler CJ, Tsai YJ, et al. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J Biol Chem*. 1994; 269:2550–2561. [PubMed: 8300583]
3. Zhu N, Liggitt D, Liu Y, Debs R. Systemic gene expression after intravenous DNA delivery in adult mice. *Science*. 1993; 261:209–211. [PubMed: 7687073]
4. Philip R, Liggitt D, Philip M, Dazin P, Debs R. *In Vivo* gene delivery: efficient transfection of T lymphocytes in adult mice. *J Biol Chem*. 1993; 268:16087–16090. [PubMed: 8344890]
5. Solodin I, Brown CS, Bruno MS, Chow CY, Jang EH, et al. A novel series of amphiphilic imidazolinium compounds for *in vitro* and *in vivo* gene delivery. *Biochemistry*. 1995; 34:13537–13544. [PubMed: 7577942]
6. Liu Y, Liggitt D, Zhong W, Tu G, Gaensler K, et al. Cationic liposome-mediated intravenous gene delivery. *J Biol Chem*. 1995; 270:24864–24870. [PubMed: 7559609]
7. Liu Y, Mounkes LC, Liggitt HD, Brown CS, Solodin I, et al. Factors influencing the efficiency of cationic-liposome mediated intravenous gene delivery. *Nat Biotechnol*. 1997; 15:167–173. [PubMed: 9035144]
8. Liu F, Qi H, Huang L, Liu D. Factors controlling the efficiency of cationic lipid-mediated transfection *in vivo* via intravenous administration. *Gene Ther*. 1997; 4:517–523. [PubMed: 9231067]
9. Thierry AR, Lunardi-Iskandar Y, Bryant JL, Rabinovich P, Gallo RC, et al. Systemic gene therapy: biodistribution and long-term expression of a transgene in mice. *Proc Natl Acad Sci U S A*. 1995; 92:9742–9746. [PubMed: 7568209]
10. Tsukamoto M, Ochiya T, Yoshida S, Sugimura T, Terada M. Gene transfer and expression in progeny after intravenous DNA injection into pregnant mice. *Nat Genet*. 1995; 9:243–248. [PubMed: 7773286]
11. Aksentijevich I, Pastan I, Lunardi-Iskandar Y, Gallo RC, Gottesman MM, et al. *In vitro* and *in vivo* liposome-mediated gene transfer leads to human MDR1 expression in mouse bone marrow progenitor cells. *Hum Gene Ther*. 1996; 7:1111–1122. [PubMed: 8773513]
12. Xu Y, Szoka FC Jr. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry*. 1996; 35:5616–5623. [PubMed: 8639519]
13. Yotnda P, Chen DH, Chiu W, Piedra PA, Davis A, et al. Bilamellar cationic liposomes protect adenovectors from preexisting humoral immune responses. *Mol Ther*. 2002; 5:233–241. [PubMed: 11863412]
14. Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD, et al. Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nat Biotechnol*. 1997; 15:647–652. [PubMed: 9219267]

15. Ramesh R, Saeki T, Templeton NS, Ji L, Stephens LC, et al. Successful treatment of primary and disseminated human lung cancers by systemic delivery of tumor suppressor genes using an improved liposome vector. *Molecular Therapy*. 2001; 3:337–350. [PubMed: 11273776]
16. Ramesh R, Ito I, Saito Y, Wu Z, Mhashikar AM, et al. Local and systemic inhibition of lung tumor growth after nanoparticle-mediated mda-7/IL-24 gene delivery. *DNA Cell Biol*. 2004; 23:850–857. [PubMed: 15684712]
17. Tirone TA, Fagan SP, Templeton NS, Wang X, Brunicardi FC. Insulinoma induced hypoglycemic death in mice is prevented with beta cell specific gene therapy. *Ann Surg*. 2001; 233:603–611. [PubMed: 11323498]
18. Shi HY, Liang R, Templeton NS, Zhang M. Inhibition of breast tumor progression by systemic delivery of the maspin gene in a syngeneic tumor model. *Mol Ther*. 2002; 5:755–761. [PubMed: 12027560]
19. Lu H, Zhang Y, Roberts DD, Osborne CK, Templeton NS. Enhanced gene expression in breast cancer cells *in vitro* and tumors *in vivo*. *Mol Ther*. 2002; 6:783–792. [PubMed: 12498774]
20. Shi Q, Nguyen AT, Angell Y, Deng D, Na CR, et al. A combinatorial approach for targeted delivery using small molecules and reversible masking to bypass nonspecific uptake *in vivo*. *Gene Ther*. 2010; 17:1085–1097. [PubMed: 20463761]
21. Liu SH, Smyth-Templeton N, Davis AR, Davis EA, Ballian N, et al. Multiple treatment cycles of liposome-encapsulated adenoviral RIP-TK gene therapy effectively ablate human pancreatic cancer cells in SCID mice. *Surgery*. 2011; 149:484–495. [PubMed: 21295812]
22. Liu S, Ballian N, Belaguli NS, Patel S, Li M, et al. PDX-1 acts as a potential molecular target for treatment of human pancreatic cancer. *Pancreas*. 2008; 37:210–220. [PubMed: 18665085]
23. Pan WH, Xin P, Morrey JD, Clawson GA. A self-processing ribozyme cassette: utility against human papillomavirus 11 E6/E7 mRNA and hepatitis B virus. *Mol Ther*. 2004; 9:596–606. [PubMed: 15093190]
24. Lu, C., et al. Systemic gene therapy with tumor suppressor TUSC2/ FUS1 nanoparticles for recurrent/metastatic lung cancer.. Annual Meeting of the American Association of Cancer Research (AACR); Orlando, FL. 2011.
25. Nemunaitis G, Jay CM, Maples PB, Gahl WA, Huizing M, et al. Hereditary Inclusion Body Myopathy: Single Patient Response to Intravenous Dosing of GNE Gene Lipoplex. *Hum Gene Ther*. 2011; 22:1331–1341. [PubMed: 21517694]
26. Nemunaitis G, Maples PB, Jay C, Gahl WA, Huizing M, et al. Hereditary inclusion body myopathy: single patient response to GNE gene Lipoplex therapy. *J Gene Med*. 2010; 12:403–412. [PubMed: 20440751]
27. Sternberg B. Morphology of cationic liposome/DNA complexes in relation to their chemical composition. *J Liposome Res*. 1996; 6:515–533.
28. Senior J, Delgado C, Fisher D, Tilcock C, Gregoriadis G. Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles. *Biochim Biophys Acta*. 1991; 1062:77–82. [PubMed: 1998713]
29. Papahadjopoulos D, Allen TM, Gabizon A, Mayhew E, Matthey K, et al. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc Natl Acad Sci USA*. 1991; 88:11460–11464. [PubMed: 1763060]
30. Gabizon A, Catane R, Uziely B, Kaufman B, Safra T, et al. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer Res*. 1994; 54:987–992. [PubMed: 8313389]
31. Gordon KB, Tajuddin A, Guitart J, Kuzel TM, Eramo LR, et al. Hand-foot syndrome associated with liposome-encapsulated doxorubicin therapy. *Cancer*. 1995; 75:2169–2173. [PubMed: 7697608]
32. Uziely B, Jeffers S, Isacson R, Kutsch K, Wei-Tsao D, et al. Liposomal doxorubicin: antitumor activity and unique toxicities during two complementary phase I studies. *J Clin Oncol*. 1995; 13:1777–1785. [PubMed: 7602367]
33. Kuai R, Yuan W, Li W, Qin Y, Tang J, et al. Targeted Delivery of Cargoes into a Murine Solid Tumor by a Cell-Penetrating Peptide and Cleavable Poly(ethylene glycol) Comodified Liposomal

- Delivery System via Systemic Administration. *Mol Pharm*. 2011; 8:2151–2161. [PubMed: 21981683]
34. Kuai R, Yuan W, Qin Y, Chen H, Tang J, et al. Efficient Delivery of Payload into Tumor Cells in a Controlled Manner by TAT and Thiolytic Cleavable PEG Co-Modified Liposomes. *Mol Pharm*. 2010
 35. Thambi T, Yoon HY, Kim K, Kwon IC, Yoo CK, et al. Bioreducible Block Copolymers Based on Poly(Ethylene Glycol) and Poly(γ -Benzyl L-Glutamate) for Intracellular Delivery of Camptothecin. *Bioconjug Chem*. 2011; 22:1924–1931. [PubMed: 21899345]
 36. Hatakeyama H, Akita H, Harashima H. A multifunctional envelope type nano device (MEND) for gene delivery to tumours based on the EPR effect: a strategy for overcoming the PEG dilemma. *Adv Drug Deliv Rev*. 2011; 63:152–160. [PubMed: 20840859]
 37. Hatakeyama H, Akita H, Ito E, Hayashi Y, Oishi M, et al. Systemic delivery of siRNA to tumors using a lipid nanoparticle containing a tumor-specific cleavable PEG-lipid. *Biomaterials*. 2011; 32:4306–4316. [PubMed: 21429576]
 38. Grosse SM, Tagalakis AD, Mustapa MF, Elbs M, Meng QH, et al. Tumor-specific gene transfer with receptor-mediated nanocomplexes modified by polyethylene glycol shielding and endosomally cleavable lipid and peptide linkers. *FASEB J*. 2010; 24:2301–2313. [PubMed: 20203088]
 39. McNeeley KM, Karathanasis E, Annapragada AV, Bellamkonda RV. Masking and triggered unmasking of targeting ligands on nanocarriers to improve drug delivery to brain tumors. *Biomaterials*. 2009; 30:3986–3995. [PubMed: 19427688]
 40. Sakurai Y, Hatakeyama H, Akita H, Oishi M, Nagasaki Y, et al. Efficient short interference RNA delivery to tumor cells using a combination of octaarginine, GALA and tumor-specific, cleavable polyethylene glycol system. *Biol Pharm Bull*. 2009; 32:928–932. [PubMed: 19420766]
 41. Gustafsson J, Arvidson G, Karlsson G, Almgren M. Complexes between cationic liposomes and DNA visualized by cryo-TEM. *Biochim Biophys Acta*. 1995; 1235:305–312. [PubMed: 7756339]
 42. Templeton NS, Lasic DD. New directions in liposome gene delivery. *Mol Biotechnol*. 1999; 11:175–180. [PubMed: 10464771]
 43. Repits J, Sterjovski J, Badia-Martinez D, Mild M, Gray L, et al. Primary HIV-1 R5 isolates from end-stage disease display enhanced viral fitness in parallel with increased gp120 net charge. *Virology*. 2008; 379:125–134. [PubMed: 18672260]
 44. Cilliers T, Nhlapo J, Coetzer M, Orlovic D, Ketas T, et al. The CCR5 and CXCR4 coreceptors are both used by human immunodeficiency virus type 1 primary isolates from subtype C. *J Virol*. 2003; 77:4449–4456. [PubMed: 12634405]
 45. Lee E, Hall RA, Lobigs M. Common E protein determinants for attenuation of glycosaminoglycan-binding variants of Japanese encephalitis and West Nile viruses. *J Virol*. 2004; 78:8271–8280. [PubMed: 15254199]
 46. Markoff L, Falgout B, Chang A. A conserved internal hydrophobic domain mediates the stable membrane integration of the dengue virus capsid protein. *Virology*. 1997; 233:105–117. [PubMed: 9201220]
 47. Reeves JD, Schulz TF. The CD4-independent tropism of human immunodeficiency virus type 2 involves several regions of the envelope protein and correlates with a reduced activation threshold for envelope-mediated fusion. *J Virol*. 1997; 71:1453–1465. [PubMed: 8995671]
 48. Andeweg AC, Boers PH, Osterhaus AD, Bosch ML. Impact of natural sequence variation in the V2 region of the envelope protein of human immunodeficiency virus type 1 on syncytium induction: a mutational analysis. *J Gen Virol*. 1995; 76:1901–1907. [PubMed: 7636471]
 49. Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc Natl Acad Sci USA*. 1989; 86:6982–6986. [PubMed: 2780554]
 50. Felgner PL, Ringold GM. Cationic liposome-mediated transfection. *Nature*. 1989; 337:387–388. [PubMed: 2463491]
 51. Pinnaduwaage P, Huang L. The role of protein-linked oligosaccharide in the bilayer stabilization activity of glycophorin A for dioleoylphosphatidylethanolamine liposomes. *Biochim Biophys Acta*. 1989; 986:106–114. [PubMed: 2819088]

52. Leventis R, Silvius JR. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim Biophys Acta*. 1990; 1023:124–132. [PubMed: 2317491]
53. Rose JK, Buonocore L, Whitt MA. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *Biotechniques*. 1991; 10:520–525. [PubMed: 1867862]
54. Loeffler JP, Behr JP. Gene transfer into primary and established mammalian cell lines with lipopolyamine-coated DNA. *Methods Enzymol*. 1993; 217:599–618. [PubMed: 8474352]
55. Simberg D, Weiss A, Barenholz Y. Reversible mode of binding of serum proteins to DOTAP/cholesterol Lipoplexes: a possible explanation for intravenous lipofection efficiency. *Hum Gene Ther*. 2005; 16:1087–1096. [PubMed: 16149907]
56. Templeton, NS., et al. Keystone Symposia on Molecular and Cellular Biology of Gene Therapy. Salt Lake City, Utah: 1999. Non-viral vectors for the treatment of disease..
57. Mislick KA, Baldeschwieler JD. Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc Natl Acad Sci U S A*. 1996; 93:12349–12354. [PubMed: 8901584]
58. Jain RK. Barriers to drug delivery in solid tumors. *Scientific American*. 1994; 271:58–65. [PubMed: 8066425]
59. Jain RK. Haemodynamic and transport barriers to the treatment of solid tumours. *Int J Radiat Biol*. 1991; 60:85–100. [PubMed: 1678003]
60. Jain RK. Transport of molecules, particles, and cells in solid tumors. *Annu Rev Biomed Eng*. 1999; 1:241–263. [PubMed: 11701489]
61. Handumrongkul C, Zhong W, Debs RJ. Distinct sets of cellular genes control the expression of transfected, nuclear-localized genes. *Mol Ther*. 2002; 5:186–194. [PubMed: 11829526]
62. Chen L, Firozi P, Barton M, Templeton NS. Widespread, exceptionally high levels of histone H3 lysine 4 trimethylation largely mediate “privileged” gene expression. *Gene Expr*. 2007; 13:271–282. [PubMed: 17605300]
63. Zhang Y, Lu H, LiWang P, Sili U, Templeton NS. Optimization of gene expression in nonactivated circulating lymphocytes. *Mol Ther*. 2003; 8:629–636. [PubMed: 14529836]
64. Baudard M, Flotte TR, Aran JM, Thierry AR, Pastan I, et al. Expression of the human multidrug resistance and glucocerebrosidase cDNAs from adeno-associated vectors: efficient promoter activity of AAV sequences and *in vivo* delivery via liposomes. *Hum Gene Ther*. 1996; 7:1309–1322. [PubMed: 8818718]
65. Gene Targeting Protocols. Humana Press; Totowa, New Jersey: 2000.
66. Woodard, LE.; Calos, MP. Nonviral genome modification strategies for gene therapy: transposon, integrase, and homologous recombination systems.. In: Templeton, NS., editor. *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies*. Third Edition. CRC Press; Boca Raton FL: 2009. p. 677-691.
67. Firozi P, Zhang W, Chen L, Quijcho FA, Worley KC, et al. Identification and removal of colanic acid from plasmid DNA preparations: implications for gene therapy. *Gene Ther*. 2010; 17:1484–1499. [PubMed: 20664542]
68. Yew NS, Zhao H, Przybylska M, Wu IH, Tousignant JD, et al. CpG depleted plasmid DNA vectors with enhanced safety and long-term gene expression *in vivo*. *Mol Ther*. 2002; 5:731–738. [PubMed: 12027557]
69. Iyer M, Berenji M, Templeton NS, Gambhir SS. Noninvasive imaging of cationic lipid-mediated delivery of optical and PET reporter genes in living mice. *Mol Ther*. 2002; 6:555–562. [PubMed: 12385291]

Types of Lamellar Vesicles

Figure 1.

Diagrams drawn from cryo-electron micrographs of cross-sections through vitrified films of various types of liposomes and DNA-liposome complexes. SUVs are small unilamellar vesicles that condense nucleic acids on the surface and produce "spaghetti and meatballs" structures. MLVs are multilamellar vesicles that appear as "Swiss Rolls" after mixing with DNA. BIVs are bilamellar invaginated vesicles produced using a formulation developed in our laboratory [14]. Nucleic acids are efficiently encapsulated between two bilamellar invaginated structures (BIVs).

Assembly of Complexes

Figure 2.

Proposed model showing cross-sections of extruded DOTAP: Chol liposomes (BIVs) interacting with nucleic acids. Nucleic acids adsorb onto a BIV via electrostatic interactions. Attraction of a second BIV to this complex results in further charge neutralization. Expanding electrostatic interactions with nucleic acids cause inversion of the larger BIV and total encapsulation of the nucleic acids. Inversion can occur in these liposomes because of their excess surface area, which allows them to accommodate the stress created by the nucleic acid-lipid interactions. Nucleic acid binding reduces the surface area of the outer leaflet of the bilayer and induces the negative curvature due to lipid ordering and reduction of charge repulsion between cationic lipid headgroups. Condensation of the internalized nucleic acid-lipid sandwich expands the space between the bilayers and may induce membrane fusion to generate the apparently closed structures. The enlarged area shows the arrangement of nucleic acids condensed between two 4 nm bilayers of extruded DOTAP: Chol.

Assembly of BIV + Adenovirus Complexes

Figure 3. Proposed model showing cross-sections of an extruded DOTAP: Chol liposome (BIV) interacting with adenovirus. Adenovirus interacts with a BIV causing negative curvature and wrapping around the virus particle.

Figure 4.
Cryo-electron micrograph of BIV DOTAP: Chol-DNA liposome complexes. The plasmid DNA is encapsulated between two BIVs.

Figure 5. Cryo-electron micrograph of extruded DOTAP:DOPE liposomes complexed to plasmid DNA. Although these liposomes were prepared by the same protocol that produces BIV DOTAP:Chol, these vesicles cannot wrap and encapsulate nucleic acids. The DNA condenses on the surfaces of the liposomes shown.

Cell Entry of Complexes

Figure 6. Mechanisms for cell entry of nucleic acid-liposome complexes. Two major pathways for interaction are by endocytosis or by direct fusion with the cell membrane. Complexes that enter the cell by direct fusion allow delivery of more nucleic acids to the nucleus because the bulk of the nucleic acids do not enter endosomes.

Figure 7. Optimized strategy for delivery and gene expression in the target cell. Optimization of many steps is required to achieve targeted delivery, shielding from non-specific uptake in non-target organs and tissues, deshielding, fusion with the cell membrane, entry of nucleic acids into the cell and to the nucleus, and production of gene expression of a cDNA cloned in a plasmid.

Figure 8. Serum stability profile for DNA-liposome complexes optimized for systemic delivery. Serum stability of these complexes was studied at 37°C out to 24 hours at concentrations of serum ranging from 0 to 100%. Serum stability at the highest concentrations of serum, about 70 to 100%, that are physiological concentrations of serum found in the bloodstream is required.