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POLYETHYLENIMINE AND LIPOSOME-BASED NON-VIRAL VECTORS FOR GENE DELIVERY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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ABSTRACT

The ultimate goal of gene therapy is to cure both inherited and acquired diseases by replacing, adding or correcting genes. Vectors designed for gene delivery can be divided into two general categories: viral and non-viral. Viral vectors use replication deficient viruses to transfer genetic material into cells. Non-viral vectors, such as polyethylenimine (PEI) and liposome-based vectors, are synthetic carriers that utilize physico-chemical methods for gene delivery. Currently, the main limitations of non-viral vectors for gene therapy are their relatively inefficient gene delivery, lack of tissue specificity, and toxicity. This dissertation outlines the development and evaluation of novel PEI and liposome-based non-viral transfection vectors designed to improve gene transfer efficiency and vector stability while reducing toxicity. Initially, using amine reactive reducible cross-linking reagents, relatively high molecular weight (HMW) PEI polymers were synthesized from low molecular weight (LMW) PEI monomers. The goal was to take advantage of the high transfection efficiency observed using transfection vectors prepared with HMW PEI, along with the decreased toxicity obtained using vectors prepared with LMW PEI. Using this strategy, efficient gene transfer was observed in vitro, and transfection related toxicity was reduced (Chapter 2). Next, in an effort to optimize transfection vectors comprised of polycation condensed DNA (polyplex) complexed with anionic liposomes, termed LPDII, a novel anionic
liposome formulation containing diolein was developed. Diolein/cholesteryl hemisuccinate liposomes released increasing amounts of encapsulated calcein and displayed a time-dependent size increase in response to decreasing pH. Furthermore, diolein-based LPDII vectors mediated improved gene expression compared with PEI-based transfection, and overcame the serum sensitivity of previous LPDII formulations (Chapter 3). Finally, diolein-based LPDII vectors were further modified by incorporating reversible covalent cross-linking, to improve vector stability, and lipid-anchored ligands, such as folate, to promote receptor-mediated internalization of transfection complexes. Cross-linking improved the stability of polyplexes used for LPDII formation, but had a negative impact on gene transfer efficiency. Inclusion of folate in the anionic liposomes used to prepare LPDII vectors led to an increase in gene expression (Chapter 4). The applications of incorporating folate into liposome formulations utilized for gene and drug delivery are also described in detail (Chapter 1).
Dedicated to my wife
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CHAPTER 1

INTRODUCTION

1.1 Preface

As knowledge accumulates in various disciplines, including chemistry, physics, biochemistry, molecular and cell biology, genetics, pharmaceutics, physiology and computer-based technologies, scientists are faced with an increasing number of options for the development and synthesis of novel therapeutic strategies. These strategies utilize rapidly growing and easily accessible information to identify new molecular targets in diseased tissues, improve therapeutic efficacy of existing treatments, and selectively treat disease by developing methods to differentiate between normal and afflicted cells. Eventually, success will be defined by strategies that take advantage of the overwhelming number of new discoveries for the logical development of advanced biotechnologies with the potential to improve prognostic and diagnostic outcomes.

Site-directed delivery of liposomes using targeting ligands is just one of many innovative biotechnologies shaping modern medicine. The application of folate receptor-targeting to liposomal drug delivery is being pursued as a strategy to selectively target cancer cells for destruction by antineoplastic drugs, or therapeutically alter the malignant phenotype by introducing exogenous genetic material. Folate receptor-targeting takes
advantage of the observation that some types of cancer consistently overexpress high levels of folate receptors, which are subsequently internalized through an endocytic pathway. Due to the high affinity with which folate binds its receptor, covalent attachment of folate to a drug particle or liposome ultimately might serve as a mechanism to discriminate between normal and neoplastic cells, while simultaneously providing a route for internalization into the cytoplasm. Ultimately, receptor-targeted drug delivery has the potential to improve drug efficacy and reduce adverse toxicities by optimizing the biodistribution of drug particles or carriers.

This review will characterize the important components and processes involved in folate receptor-mediated delivery of liposomes. It will begin with a discussion on folate and mechanisms for folate uptake into tumor cells. Then, a description of liposomal drug carriers will be provided, followed by a comprehensive overview of folate receptor-targeted liposomes for the delivery of drugs and nucleic acids. The conclusion will consist of a brief consideration of where the field of folate receptor-targeted liposomal drug delivery may be heading and potential obstacles to future progress.
1.2 Folate

Folic acid (folate, pteroylglutamic acid) is an essential water-soluble vitamin of the B-complex group. Mammals are incapable of synthesizing folate, and therefore metabolic needs must be satisfied through dietary intake of the vitamin. Chemically, folate is comprised of three distinct moieties: 6-methylpterin, p-aminobenzoic acid and L-glutamic acid [1] (Figure 1.1). Folate exists in several oxidation states, and is converted intracellularly to the biologically active 5,6,7,8-tetrahydrofolate (THF) through the enzymatic action of dihydrofolate reductase. Interestingly, classical anti-folate compounds such as methotrexate (MTX) function by competing for this enzyme, ultimately inhibiting substrate binding and depriving cells of their metabolic requirements for reduced folate compounds.

THF is further adapted intracellularly by the addition of a polypeptide tail consisting of multiple glutamic acid residues. This polyglutamation serves to sequester the newly formed polyanion inside the cell, and generally increases its affinity for the various intracellular folate-binding enzymes [2]. The nitrogen atoms at positions 5 and 10 are common reactive sites of the THF molecule, and function individually or collectively as attachment points for methyl (−CH₃), methylene (−CH₂−), formyl (−CH=O), formimino (−CH=NH) or methenyl (−CH=) groups [1]. The physiological form of folate in human serum is 5-methyltetrahydrofolate (5-MeTHF), and is present at a concentration of ~ 5-30 nM. Reduced folates participate as coenzymes in a number of important biochemical pathways involving the mobilization and utilization of one-carbon units. Among these are the biosynthesis of thymine from uridine, the de novo synthesis of purines, the conversion of homocysteine to methionine and participation in serine and glycine
metabolism [1]. Folate deficiency is associated with an increased risk for cardiovascular disease [3], cancer, cognitive defects [4,5] and congenital malformations during pregnancy [6].

As a targeting ligand, folate has several advantages: (1) folate is a relatively small hydrophilic molecule (441 Da), which will not significantly hinder aqueous solubility or diffusion, (2) the conjugation chemistry is simple and defined, and covalent conjugation of folate by its γ-carboxyl group does not significantly hinder receptor binding (3) folate is conveniently available in large quantities of pure compound, avoiding potentially laborious and expensive procedures involved in antibody preparation and isolation, (4) folate has low immunogenicity, thus avoiding concerns of clearance by the immune system or activation of an immune response after repeated administration, (5) folate is a stable molecule, resistant to degradation under adverse storage or reaction conditions and (6) folic acid has a high affinity for the folate receptor, which is selectively overexpressed in some types of cancer.
1.3 Folate transport in tumor cells

1.3.1 Characterization of the reduced folate carrier and high affinity folate receptors

Two distinct transport systems mediate folate acquisition by cancer cells, the one-carbon, reduced-folate carrier (RFC) and the high affinity folate receptor (FR). The reduced folate carrier is a 46 kDa integral membrane protein, and a potential member of the 12-transmembrane domain-spanning transporter family [7,8]. The RFC is a high capacity, low affinity transport system involved in the primary transport of reduced folates in some neoplastic cells. The transporter is characterized by its relatively higher affinity for 5-substituted reduced folates and methotrexate (K\text{m} \sim 1-10 \, \mu\text{M}) compared with folic acid (K\text{m} \sim 50-200 \, \mu\text{M}) [7,9]. Although the reduced folate carrier is an important route for the internalization of folates and anti-folate compounds, especially at pharmacological concentrations (\mu\text{M}), it does not appear to function in drug targeting due to its relatively low affinity for folic acid.

Alternatively, folate receptors [reviewed in 8,10-12], or folate binding proteins (FBP), are characterized by their high affinity for folic acid (K\text{d} \sim 0.1-1 \, \text{nM}) and 5-MeTHF (K\text{d} \sim 1-10 \, \text{nM}), with relatively lower affinities for methotrexate and other folate compounds [10, 13]. Folate binding proteins were originally isolated as \sim 38-40 \, \text{kDa} glycoproteins with an amino acid content of \sim 28 \, \text{kDa}. Three isoforms of the FR have since been identified in humans, FR-\alpha [14-16], FR-\beta [15] and FR-\gamma/\gamma' [17-18]. The \gamma' isoform is differentiated from FR-\gamma by the loss of 138 C-terminal amino acids resulting in a truncated 81-residue polypeptide, and originates from a two base deletion leading to a stop codon in the cDNA of FR-\gamma. The remaining full-length folate receptors exhibit 68-79\% homology with respect to their amino acid sequence, and range in length from 220
to 236 amino acids [19]. Folate receptors contain either two (β and γ) or three (α) sites for N-glycosylation and retain 16 conserved cysteine residues in their peptide sequences, which may participate in disulfide bond formation for the stabilization of receptor tertiary structure. Glycosylation is important for the proper folding of the nascent polypeptides, intracellular trafficking and ultimately surface expression levels of functional receptors, but is not required for folate binding [20]. FR-α and β are attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor [21,22], whereas FR-γ/γ' is constitutively secreted due to lack of an efficient signal for GPI modification [13]. Interestingly, α and β receptor isoforms exhibit opposite stereospecificities and different affinities for various folate compounds [19,23,24]. The α isoform preferentially binds physiological (6S) folate coenzymes, while FR-β shows a greater affinity for the non-physiological (6R) isomers. In addition, it has been shown that FR-α binds common folate coenzymes and anti-folates with 2-100 fold greater affinity than FR-β [23].

1.3.2 Tissue distribution and expression of folate receptors

The distribution and expression of folate receptors in normal and neoplastic tissues and cell lines has been well documented [25-29]. FR-α is highly expressed in normal placenta, kidney (proximal tubules), fallopian tubes and choroid plexus [30], with more moderate expression in some normal cells of the lung, breast and thyroid [31,32]. The α isoform is a marker for ovarian cancer due to its consistent and high levels of overexpression [33-36], and is also often overexpressed in uterine carcinomas [37]. Less consistent overexpression of FR-α has been observed in tumors of the colon, kidney,
breast, lung, brain and testis. FR-β is expressed in low levels on most normal cells, with more moderate levels in colon, spleen, thymus and placenta. It has also been suggested that the β isoform is a neutrophilic lineage marker, and has been shown to be differentially expressed in certain subtypes of myeloid leukemia [38,39]. Expression of the γ isoforms is largely restricted to certain normal and malignant hematopoietic tissues, such as the spleen, thymus and bone marrow, although FR-γ/γ' have been detected in carcinomas of the ovary, uterus and cervix as well [13,17]. An appropriate generalization for receptor expression is that FR-α is often overexpressed in malignancies of epithelial origin, whereas FR-β has a tendency for overexpression in malignancies of non-epithelial origin, and FR-γ/γ' are mainly expressed at low levels by certain hematopoietic cells. It should be noted that soluble forms of the FR exist as well [40,41], but their characterization has been intentionally neglected due to their insignificant role in folate receptor-targeting.

1.3.3 Folate receptor-mediated endocytosis

Several lines of evidence support a direct role for folate receptors in the binding and internalization of folate compounds [42-45], but some ambiguity remains concerning the mechanism(s) for folate receptor-mediated endocytosis. There is little doubt that folate compounds bind membrane-associated folate binding proteins with high affinity for subsequent internalization, but several questions linger concerning the diffuse or punctate distribution of folate receptors at the cell surface, their localization on the plasma membrane and ultimately the translocation mechanism of folate compounds into the
cytoplasm. The popular model proposed to describe the mechanism for folate internalization is termed potocytosis, so called in order to differentiate it from the classical clathrin-dependent endosomal pathway [46]. This model predicts the association of FR-clusters around or within small invaginations in the plasma membrane called caveolae or plasmalemmal vesicles. Caveolae are small (~ 60 nm), omega (Ω)-shaped invaginations in the plasma membrane, with a distinctive cytoplasmic coat of striated thin filaments composed, in part, of the protein caveolin (VIP21) [47]. Following folate binding, these caveolae are thought to transiently seal folate-receptor complexes within membrane-attached vesicles. Due to the pH-lowering activity of membrane associated proton pumps, folate then dissociates from its receptor and is transported down its concentration gradient by a putative anion transporter into the cytoplasm. Finally, the caveolae reopen with vacant folate receptors, ready to participate in another round of folate internalization [48,49].

The potocytosis model garners partial support from experiments that show colocalization of GPI-anchored proteins and caveolin-rich membrane domains in detergent-insoluble membrane fractions [50], and studies which demonstrate that both caveolae and FR-clusters are dependent on the presence of cholesterol [51]. In fact, inhibition of potocytosis, and folate internalization, has been accomplished by disruption of caveolae using cholesterol-binding drugs, such as filipin or nystatin, or protein kinase C activators, such as PMA (phorbol-12-myristate-13-acetate) [52,53]. Further support is offered from studies that localize folate receptors in or near caveolae using electron microscopy of folate targeted bovine serum albumin-colloidal gold (BSA-CG) conjugates [54], although these same studies simultaneously contradict the potocytosis model by showing that
labeled protein conjugates later appear in tubular endosomes and multivesicular bodies. Other conflicting reports indicate that receptor clustering and co-localization of folate receptors with caveolin-rich membrane domains are experimental artifacts, which do not accurately represent endogenous receptor localization on the cell surface. In short, the existence of data that partially or completely contradict observations and conclusions drawn from the studies cited above cast justifiable doubt on the potocytosis mechanism [55-58], and the definitive folate receptor-mediated internalization pathway(s) remains to be elucidated.

Regardless of this debate, for the purpose of folate targeted drug delivery it has been convincingly demonstrated that folate attached as a targeting ligand facilitates the nondestructive internalization of a variety of molecules. This point is conveniently illustrated by studies that demonstrated folate targeting of several proteins [59]. Leamon and Low showed that folate conjugated to ribonuclease, horseradish peroxidase (HRP), BSA or IgG allowed intracellular delivery of $>10^6$ copies of these macromolecules within a two-hour period. Furthermore, their data confirmed that this uptake was folate receptor-mediated based on several observations. First, they observed binding and internalization of folate-labeled fluorescent proteins in receptor positive KB cells, whereas unlabeled proteins were not internalized. They also showed that even under conditions of acid-saline washing (conditions known to dissociate folate from its receptor), that folate-conjugated proteins could not be entirely removed from cells (indicating internalization), but non-targeted proteins were easily removed with a simple saline wash. Moreover, binding and uptake of folate-labeled proteins could be competitively blocked with excess free folate or antibodies reactive towards the folate
receptor. These studies also demonstrated that folate receptor-mediated delivery was nondestructive by detecting exogenous HRP activity inside targeted cells, whereas HRP activity was undetectable using enzymes without folate derivatization. Finally, the authors suggest that even relatively large proteins such as ferritin, with a molecular weight of 443 kDa, could be internalized via this pathway, suggesting that conjugate size is relatively unimportant below this level.

These studies have since been extended to include a variety of folate receptor-targeted molecules, including cytotoxic proteins like momordin and *Pseudomonas* exotoxin [60,61], chemotherapeutic drugs like maytansinoids [62], anti-T-cell receptor antibodies [63], folate-targeted enzymes for prodrug therapy [64], radioimaging agents [65-69], antisense oligodeoxyribonucleotides [70,71], viral and non-viral gene delivery vectors [72-74] and others. A surveying of this partial list demonstrates the versatility and utility of targeting approaches directed at the folate-receptor. Unfortunately, detailed analyses of each of these folate receptor-targeted therapeutic and diagnostic agents is beyond the scope of this review, but have been thoroughly reviewed elsewhere [75-78]. This review will focus on applications of folate receptor-targeted liposomes for the delivery of drugs and nucleic acids.
1.4 Liposomes

1.4.1 Characterization of liposome structure and properties

Liposomes are small vesicles composed primarily of amphipathic phospholipids, such as phosphatidylcholine (PC) or phosphatidylethanolamine (PE) [reviewed in 79-82]. These phospholipid molecules are composed of polar head groups conjugated to non-polar fatty acyl-, alkyl- or alkoxy-hydrocarbon tails, which may vary in length and degree of saturation. When exposed to an aqueous environment, most phospholipids have a tendency to spontaneously self-associate to achieve an orientation that is thermodynamically favorable. This association is dependent on several factors, including the properties of the lipid component and its concentration, temperature, pH, and ionic strength of the buffer. Aggregation of the hydrocarbon regions, so as to exclude the aqueous solvent, can ultimately lead to the formation of liposomes. Four criteria are generally used to characterize liposomes: size, number of lamellae, charge and stability, all of which can be manipulated and controlled by varying preparation materials and/or methods.

The diameter of liposomes can vary anywhere between ~30 nm to several micrometers, but for the purposes of drug delivery, liposomes are usually smaller than 150 nm. Common preparation methods for liposomes, including the reverse-phase evaporation method used in our laboratory, often yield multilamellar liposomal vesicles. Multilamellar vesicles are composed of multiple concentric lipid bilayer spheres, whereas unilamellar vesicles are comprised of a single membrane bilayer (Figure 1.2). In order to obtain unilamellar vesicles with a relatively uniform size distribution, preparations containing heterogeneously sized liposomes with variable numbers of lamellae are often
extruded several times through polycarbonate membranes with fixed pore diameters. This procedure primarily yields unilamellar liposomes with an average diameter slightly larger than the pore size of the membrane (~20-40%).

The surface charge on liposomes is mainly the result of variations in the phospholipid headgroups used for liposome preparation, although pH is also a factor, and liposomes may be positively or negatively charged depending on the net lipid composition. Surface charge can be important in stabilizing some particular lipid conformations, and can also influence pharmacokinetic properties, including clearance. Similarly, liposome stability is also influenced by variations in liposome formulation, and may be controlled with additives such as cholesterol (Chol). Stability is often characterized by factors such as circulation half-life ($t_{1/2}$), systemic clearance, drug release rate or susceptibility to lipid exchange with circulating plasma proteins. In general, using lipids with high gel-to-liquid crystalline phase transition temperatures ($T_m$), introducing cholesterol in the lipid formulation and/or coating liposomes with hydrophilic moieties, such as carbohydrates, glycolipids or poly(ethylene glycol) (PEG), increases the plasma $t_{1/2}$ while reducing drug release rates and systemic clearance by the reticuloendothelial system (RES) [83-87].

Liposomes have been shown to induce activation of the complement system. However, incorporation of a PEG coating on the liposome surface is thought to create a steric barrier to prevent insertion of complement opsonins into the liposome bilayer (Figure 1.3) [88,89]. This, in turn, reduces recognition and uptake by phagocytic cells in the spleen and liver, and by circulating macrophages in the bloodstream (components of the RES). Using lipids with a high $T_m$ and incorporating cholesterol in the lipid membrane lead to more rigid bilayers, which may decrease drug leakage and prolong
circulation in the plasma. However, incorporation of PEG, either during or after liposome formation, has become more important in terms of increasing circulation $t_{1/2}$, thus allowing utilization of more fluid bilayer formulations for altered drug release characteristics without dramatically compromising liposome stability in plasma.

Common formulations for sterically stabilized liposomes (those including PEG) generally include 4-6 mol % PEG derivatized PE and ~ 35 mol % Chol, with the remainder being high $T_m$ lipids like HSPC (hydrogenated soy PC) or DSPC (distearoyl-PC). Conventional liposomes, without a PEG coating, often consist of DSPC:Chol at 55:45 or 66:33 molar ratios, or egg PC:Chol at a molar ratio of 3:2 (egg PC is a heterogeneous lipid mixture with a low $T_m$).

1.4.2 Liposomes as drug carriers

Liposomes have been used for the delivery of chemotherapeutic agents, antimicrobial compounds, as immunological adjuvants in vaccines, in diagnostic imaging and for the ocular delivery of drugs. They were first implemented as an injectable therapy in humans in 1974, when they were used to deliver the antibiotic amphotericin B (AmBisome®) [80]. Since then, numerous drugs and compounds have been incorporated into liposomes [90-94], aided by the development of optimized liposome formulations and a detailed understanding of their biological and pharmacokinetic properties.

Folate receptor-targeted liposomes were developed with the intent of treating various malignancies overexpressing the folate receptor. However, several inherent advantages of liposomal drug delivery augment this targeting strategy. First, liposomes are versatile in the types of compounds that can be delivered, and include lipophilic, hydrophilic and
amphipathic drugs. Hydrophilic drugs are encapsulated in the aqueous interior of liposomes during liposome formation, while hydrophobic drugs preferentially associate with the phospholipid membrane (Figure 1.3). Amphipathic drugs are unique in that they are weak acids or weak bases, and although they are similarly incorporated in the aqueous interior of liposomes, 'remote loading' procedures based on pH or ammonium sulfate gradients have been developed to achieve encapsulation efficiencies of drug in excess of 98% [95]. Interestingly, the same properties that allow efficient encapsulation of amphipathic drugs inside liposomes will also affect their release inside endosomal compartments where the pH is ~ 5.0 [96]. Furthermore, liposomes are non-toxic at pharmacological doses, and are biodegradable as a result of most liposome components being naturally occurring phospholipids. Liposome encapsulation also provides protection to drugs or nucleic acids, which may otherwise be susceptible to degradation by plasma proteases and nucleases, or become highly bound by circulating proteins. Finally, liposomes often exhibit pharmacokinetic properties that are radically different from those of the encapsulated drug, often improving the therapeutic index of highly toxic compounds, such as those utilized for cancer chemotherapy [97,98].

Since it is widely employed as an anticancer agent and has been well characterized [99-102], the pharmacokinetic differences between conventional therapeutic agents and liposomal drugs can be illustrated using the anthracycline doxorubicin (DOX) as an example. Currently, two liposomal DOX formulations (Doxil® and EVACET®) and a DOX analog, daunorubicin (DaunoXome®), have been approved for human use and are being evaluated for cancer chemotherapy [82]. Although DOX is highly effective against a variety of tumors, when administered intravenously this drug assumes a large volume of
distribution ($V_d$) and is cleared relatively rapidly from the circulation. In effect, drug concentration at the tumor site is relatively low, while normal tissues experience significant toxicity. This toxicity is manifested as nausea, alopecia, skin reactions and myelosuppression, which is dose limiting. By encapsulating DOX in liposomes, which are primarily confined to the circulation, the $V_d$ is dramatically reduced, thus limiting drug exposure to normal tissues and reducing systemic toxicities. In addition, due to the leaky microvasculature, poor lymphatic drainage and high interstitial pressure in tumors, liposomes are able to extravasate and preferentially accumulate in tumors, potentially leading to higher drug concentrations. Several clinical trials involving the use of liposomal DOX have documented a decrease both in the incidence and severity of the adverse side effects discussed above [103-111]. Finally, some acute toxicities are dependent on the peak concentration of drug, and liposomal drug formulations often reduce peak concentrations while achieving similar or improved overall drug exposure to diseased tissues.

1.4.3 pH-Sensitive liposomes

A common misconception regarding drug delivery using liposomes is that internalization of drug molecules is mediated by the fusion of the cell membrane and the liposome bilayer. However, if membrane fusion were to take place using common preparation materials, the liposome formulation itself would be inherently unstable as a result of liposomes recombining to form larger liposomes. In reality, in the absence of cellular internalization, liposomes function as controlled release devices for therapeutic
agents, with drug release depending largely on the lipid composition of the bilayer membrane.

As discussed, factors that increase liposome stability in the circulation often decrease the rate at which drugs are released. This problem necessitates a balance between liposome stability and drug release in order to achieve therapeutically relevant drug concentrations at the diseased site. Although liposomes are generally not internalized in the absence of a targeting ligand, their ability to accumulate near tumors via extravasation through discontinuous endothelia with relatively large fenestrae often results in the effective treatment of various malignancies. Despite this success, a large fraction of the dose remains entrapped inside liposomes, which are ultimately cleared from the body before they can exert any therapeutic effect.

To circumvent this problem, pH-sensitive liposome formulations have been developed in an attempt to facilitate intracellular drug release at the site of action. This strategy relies on pH differences between plasma (~7.4) and endosomal compartments (~5.0), and therefore mandates cellular internalization of liposomes to be effective. pH-sensitive liposome formulations are generally composed of dioleoylphosphatidylethanolamine (DOPE) in combination with weakly acidic lipids or cholesterol derivatives with a titratable head group. Examples of such lipids include oleic acid (OA), palmitoylhomocysteine (PHC), cholesterol hemisuccinate (CHEMS) or dipalmitoylsuccinylglycerol (DPSG) [112,113]. However, pH-sensitivity has also been achieved with liposome formulations containing diplasmenylcholine (DPPlsC) [114], N-Citraconyl-PE [115,116] and by incorporating polypeptides that undergo conformational changes at low pH to destabilize plasma membranes and facilitate drug release [117,118].
The mechanism underlying this pH-sensitivity using DOPE arises from inherent characteristics of the phosphatidylethanolamine lipid component. Due to its small headgroup and relatively larger hydrocarbon region, DOPE has an overall molecular shape of an inverted cone, and at physiological pH assumes the type two inverted hexagonal phase lipid conformation (H_{II}) (Figure 1.4). However, by incorporating a weakly acidic negatively charged lipid, DOPE is capable of forming the more well-known L\textsubscript{α} bilayer phase at physiological pH, presumably as a result of increased electrostatic repulsion between negatively charged residues and a decrease in hydrogen bonding between the ethanolamine headgroups. When exposed to the low pH of the endosomal compartment, the weakly acidic functional groups will be protonated, thus allowing PE to assume its native conformation (H_{II}). This conformational change results in the release of liposome contents and the subsequent destabilization of the endosomal membrane, which ultimately facilitates cytoplasmic release of the internalized cargo. This strategy is particularly amenable to folate receptor-targeting because folate is a ligand that mediates liposome internalization into acidic endosomal compartments, which is an obvious prerequisite for the implementation of this approach. However, a major disadvantage of pH-sensitive liposomes is that many formulations lose their pH-sensitive properties in the presence of serum, which may limit their application in vivo.
1.5 Folate receptor-targeted liposomes for drug delivery

Folate receptor-targeted liposomes [also reviewed in 119,120] incorporate many of the advantages previously discussed for non-targeted liposome formulations, including reduced toxicity and extended systemic circulation time of the encapsulated drug. In addition, this strategy also offers several advantages over the direct conjugation of folate to individual therapeutic or diagnostic drug molecules. For example, many molecules lose their biological activity upon conjugation to folate, or alternatively may not contain easily accessible functional groups for folate attachment. In addition, liposome encapsulation promotes delivery of multiple drug molecules at each receptor site, therefore increasing the amount of drug for internalization, potentially leading to a greater therapeutic effect. Furthermore, due to the multivalent nature of attachment to the cell surface, folate-conjugated liposomes will likely have a stronger affinity for receptor expressing cells, compared with molecules derivatized with a single folate ligand. This property stems from the fact that the association constants of multivalent attachments approximate the product rather than the sum of each individual binding event. Finally, liposome encapsulation prevents accumulation in normal tissues with endogenous folate receptor expression. This is achieved by preventing glomerular filtration by the kidneys, and sequestering liposomes in the circulation where apically oriented folate receptors are inaccessible.

However, there are some potential disadvantages regarding the use of folate-conjugated liposomes for drug delivery. First, due to their relatively large size, liposome diffusion through tumors may be a slow process, and penetration of liposomes into the interior of tumors will be limited by the high interstitial pressure characteristic of this
region. Furthermore, only those cells that are readily perfused will be easily accessible for treatment, and small tumors may be poorly perfused or avascular, thus preventing liposome accumulation. Additional factors that may influence treatment success involve the pore size of tumor endothelia or a phenomenon known as the 'binding site barrier'. Pore size has been shown to be relatively unimportant, as long as the pores are significantly larger than extravasating particles [121], but this may not always be the case when using liposomes for drug delivery. The binding site barrier is concerned with the idea that targeted complexes will bind those cells which they first encounter near the blood supply, but will ultimately limit drug exposure by inhibiting binding and penetration of subsequently delivered particles [122]. Finally, folate receptor expression is likely heterogeneous, both spatially and temporally, within individual and between different tumor masses leading to variable targeting efficiency.

1.5.1 Incorporation of folate as a targeting ligand on liposomes

There are two strategies to accomplish folate conjugation for subsequent liposome targeting. Either folate is attached to preformed liposomes, or alternatively, folate-derivatized lipids may be incorporated during liposome preparation. The latter is the preferred method due to the limitations associated with the former conjugation approach. These limitations include liposome exposure to potentially unfavorable reaction conditions, such as organic solvents or other hydrophobic reagents, which could potentially compromise liposome integrity. Furthermore, linking folate to preformed liposomes may result in inconsistent and uncontrollable labeling, potentially altering the liposome surface properties, resulting from unreacted lipid molecules activated for folate
attachment. Alternatively, folate is insensitive to denaturation by organic solvents, and therefore synthesis of folate-derivatized lipids does not suffer from the complication of instability. Moreover, since it is possible to purify lipids that have been successfully labeled with folate, the fraction of folate-conjugated lipids in any liposome formulation can be precisely controlled. However, since lipids are randomly incorporated into the bilayer membrane, folate will be present on both the luminal and peripheral liposome surfaces when utilizing this labeling method.

Another important issue to consider when conjugating folate to liposomes is the insertion of a PEG spacer between the targeting ligand and the liposome surface. Studies have shown that cellular uptake of fluorescent unilamellar liposomes by cultured KB cells (a FR⁺ human nasopharyngeal epidermal carcinoma) is critically dependent on the presence and length of a PEG spacer. The spacer presumably facilitates folate interaction with folate receptors on the cell surface by decreasing steric hindrance and potentially allowing multivalent attachments due to the flexible nature of the PEG molecule [123,124]. Results demonstrate that the number of liposomes internalized by cells treated with constructs containing 250 Å PEG spacers (Mr ~ 3350) was 37-fold greater than liposomes constructed with 23 Å spacers, which in turn was not significantly different from non-specific uptake of liposomes without folate derivatization [123].

The most common lipid anchor utilized to mediate folate targeting of liposomal drug carriers is distearoylphosphatidylethanolamine (DSPE), with a 250 Å PEG spacer separating folic acid from the DSPE molecule (folate-PEG3350-DSPE). Alternatively, our lab has recently shown that folate linked to cholesterol via a 250 Å PEG spacer may also be utilized for incorporation into the liposome bilayer to mediate folate receptor-
targeted delivery [125] (Figure 1.5). It has been shown that inclusion of as little as 0.1 mol % folate-derivatized lipids in a liposome formulation is sufficient to mediate efficient cellular uptake in vitro.

1.5.2 Delivery of Doxorubicin using folate-conjugated liposomes

Preliminary studies by Lee and Low evaluating folate receptor-targeted delivery of liposomes to KB cells in culture utilized the fluorescent dye calcein for encapsulation. As discussed, these studies demonstrated the need for a long PEG spacer between the liposome and folate moiety for efficient cellular internalization, but they also clearly demonstrated the importance of folate in mediating cellular uptake of liposomes (Figure 1.6) [123]. Fluorescent images in the right panels of Figure 1.6 show the sharp contrast in calcein internalization 4 h after treatment with folate-derivatized liposomes (bottom), as compared with conventional liposomes (top). By comparing these images, it is readily apparent that calcein uptake was not observed in the absence of folate conjugation. Furthermore, competitive inhibition of targeted liposomes with excess free folate, or inhibiting receptor binding by employing antiserum raised against the folate receptor, confirmed the involvement of folate in this internalization process. Surprisingly, 0.2 mM folate was required to reduce cellular uptake of folate conjugated liposomes by 50%, despite the fact that the $K_d$ for folate is <1 nM. This highlighted the avid binding that can be achieved with folate targeting, and suggested that endogenous serum folate should not affect liposome binding, since it is usually present at a concentration below 20 nM. Finally, although the kinetics were slower and the maximum receptor occupancy decreased using folate receptor-targeted liposomes, compared with the studies evaluating
folate receptor-targeted proteins discussed above, the size limit for internalization via folate receptor-mediated endocytosis was increased to include liposomes with a mean diameter of 66 nm. The authors suggest that slower uptake of liposomal carriers is a function of their larger size, which could account for the decrease in receptor binding as well, due to receptor occlusion and limited surface area of the cell membrane.

To extend these studies to determine if these results would translate into a therapeutic advantage, doxorubicin was encapsulated in several liposome formulations and administered to KB cell monolayers. Liposome formulations were composed of DSPC/Chol at a molar ratio of 56:40 either in the presence or absence of 4 mol % PEG (M_r ~ 2000) coating and/or 0.1 mol % folate-PEG3350-DSPE. Several observations concerning the advantages of DOX delivery using folate receptor-targeted liposomes were obtained [126]. It was demonstrated that uptake of folate-conjugated liposomal DOX was 45-times higher than DOX delivered by unconjugated liposomes, and 1.6-times higher than free DOX. In addition, the cytotoxicity was 86-fold and 2.7-fold greater, respectively. Although differences in cellular uptake and cytotoxicity between folate-conjugated liposomes and free DOX are relatively minor, Gabizon et al. reported that free DOX is cleared 450-times faster than DOX encapsulated in sterically stabilized PEG-coated liposomes \textit{in vivo}. This observation highlights the point that \textit{in vitro} cytotoxicity studies do not offer a fair comparison between free DOX and folate-conjugated liposomal DOX, due to the absence of any clearance component. Interestingly, a recent study by Goren et al. also indicates that encapsulating DOX in folate receptor-targeted liposomes offers a mechanism to bypass the multidrug-resistance efflux pump (P-gp), which is responsible for decreasing the effectiveness of several...
chemotherapeutic regimens [127]. Incorporation of 4 mol % PEG coating on the liposome surface did not interfere with liposome uptake or cytotoxicity. However, this observation conflicts with a more recent report by Gabizon et al., which states that receptor binding by folate was significantly inhibited by incorporation of a PEG coating [124].

Lee and Low demonstrated the specificity of folate receptor-targeted drug delivery by treating folate receptor-positive HeLa cells (a human cervical carcinoma) in co-culture with folate receptor-negative W138 cells (a human lung fibroblast) (Figure 1.7). The fluorescent images in Figure 7 compare the difference in selective cytotoxicity using folate-conjugated or unconjugated liposomes for DOX delivery. The middle panels show that after a 72 h treatment of co-cultures with non-targeted liposomal DOX (10 μM) that the smaller more aggressive HeLa cells eventually overtake the W138 cells in culture, similar to the situation observed with the untreated control in the left panels. In contrast, the right panels dramatically show that cultures treated with folate receptor-targeted liposomal DOX selectively kill receptor positive HeLa cells, primarily leaving W138 cells 72 hours after treatment. Finally, uptake was shown to be folate receptor-mediated by inhibiting DOX internalization with 1 mM free folate.

1.5.3 Delivery of Ara-C using pH-sensitive folate receptor-targeted liposomes

The advantages of folate conjugation were also extended to combine the benefits of pH-sensitive liposome formulations for enhanced endocytic drug release. Rui et al. encapsulated 1-β-D-arabinofuranosylcytosine (AraC) in pH-sensitive diplasmenylcholine (DPPlsC) folate-conjugated liposomes for subsequent administration to KB cell
monolayers [114]. The cytotoxic effect was measured by evaluating the percent of $[^3\text{H}]$-thymidine incorporation into DNA over a 24 h incubation period following a 4 h treatment. These authors compared diplasmenylcholine liposomes composed of DPPIsC/DHC (dihydrocholesterol)/DSPE-PEG3350-folate prepared at a ratio of (9:1:0.05), with egg PC/Chol/DSPE-PEG3350-folate liposomes (6:4:0.05) and free AraC. Although no IC$_{50}$ value was provided for folate-targeted pH-insensitive liposomes composed of egg PC, these authors did report a ~ 6000-fold enhancement of DNA synthesis inhibition in cells treated with folate-conjugated DPPIsC liposomes, compared with free AraC (IC$_{50} = 490$ nM and 2.8 mM for DPPIsC/folate liposomes and free AraC, respectively). From data comparing the two liposome formulations and free drug, it is also apparent that the pH-sensitive liposome formulation was significantly more effective in reducing DNA synthesis than liposomes without a pH-sensitive component. Furthermore, including DHC in the DPPIsC liposome formulation appeared to offer improved stability, with <5% drug release after 48 h in the presence of 50% serum (pH 7.4, 37 °C).
1.6 Folate receptor-targeted liposomes for the delivery of nucleic acids

Gene therapy is an emerging biotechnology that has the potential to cure many inherited and acquired diseases through the introduction of exogenous genetic material. Unlike conventional therapeutic agents, which often treat the symptoms of a disease, gene therapy has the potential to abolish the etiology of genetic disorders by introducing functional nucleic acid sequences into the nuclei of afflicted cells. Currently, the primary obstacle for effective gene therapies is the lack of an efficient system for intracellular gene delivery. Some success has been attained using genetically modified viruses as gene delivery vectors, but concerns regarding their immunogenicity, limited cargo capacity, laborious preparation procedures and the potential for insertional mutagenesis has led scientists to search for alternative means to deliver genetic material. The success of liposomes as drug carriers, and the ability to easily tailor formulations that are variable in size, charge and targeting capacity, has led to their implementation as vectors for gene therapy [128,129].

Early strategies utilizing liposomes for gene transfer employed liposomes composed of lipids or cholesterol derivatives with monovalent or polyvalent cationic headgroups [130-132]. These lipids subsequently form liposomes with a net positive charge capable of interacting with DNA through electrostatic interactions. The structural features of these complexes are variable, but visualization using freeze-fracture electron microscopy has revealed aggregated and fused liposomes interspersed among tubular rod-like structures, believed to be supercoiled DNA rods covered by a single lipid bilayer membrane. However, the associations between cationic liposomes and DNA molecules often result in complexes characterized by an overall positive charge, preventing the use
of targeting strategies due to non-specific cellular uptake by electrostatic interactions between these complexes and the negatively charged cell membrane. Therefore, for folate receptor-targeting to be effective, two general strategies have been employed. The first method involves direct liposome encapsulation of antisense oligodeoxyribonucleotides, and the second approach introduces cationic polymers to condense DNA prior to liposome incorporation.

1.6.1 Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor

The first report of folate-targeted liposomes being used to specifically mediate the internalization of nucleic acids appeared in 1995 [133]. Wang et al. evaluated the effect of encapsulating antisense oligodeoxyribonucleotides inside egg PC/Chol/folate-PEG3350-DSPE (3:2:0.5, m/m) liposomes, or in their non-targeted counterpart. The authors used several oligonucleotides, including a phosphodiester 15-mer antisense to the stop codon in the epidermal growth factor receptor (EGFR) gene, the same sequence with three phosphorothioate linkages at each terminus, two control oligonucleotides uncomplimentary to the EGFR stop codon and fluorescent homologs of several of the above. The purpose of incorporating the phosphorothioate linkages was to increase the stability of the oligonucleotides, since modifications of the phosphodiester backbone confer resistance to enzymatic degradation. In these experiments, KB cell monolayers were treated for 4 h with 3 μM oligodeoxyribonucleotide, and the effect on cell proliferation was measured 48 h after treatment.
The results from this study showed that both the phosphodiester and the phosphorothioate analog mediated virtually identical growth inhibition, reducing KB cell proliferation by >90% when encapsulated in folate-conjugated liposomes. The remaining oligonucleotides were generally much less effective in mediating growth inhibition (<10%), as were oligonucleotides incorporated in non-targeted liposomes or when administered as unencapsulated nucleotides (≤ 20%). Measurements of cellular uptake of fluorescein-labeled oligonucleotides confirmed that folate-PEG-DSPE liposomes mediated the highest levels of nucleotide internalization. Furthermore, a near quantitative reduction of EGFR was observed in cells treated with antisense phosphodiester oligonucleotides encapsulated in folate receptor-targeted liposomes, as measured by immunofluorescent labeling of the EGFR using a monoclonal antibody raised against the receptor. In summary, although no advantage was detected using phosphorothioate oligodeoxyribonucleotides, the benefits of encapsulation in folate-conjugated liposomes were clearly demonstrated.

1.6.2 Gene delivery using liposome-entrapped polycation condensed plasmid DNA: LPDI and LPDII

Although it is clearly feasible to encapsulate small oligonucleotides inside liposomes, plasmid DNA is a polyanion that can have a hydrodynamic diameter of up to 200 nm, which by itself exceeds the optimal diameter of particles for gene transfer. To overcome this physical limitation, Gao and Huang hypothesized that incorporating polylysine with a cationic lipid formulation composed of DOPE/1-β-[N-(N',N'-dimethyl-aminoethane)-carbamoyl]cholesterol (DC-chol) (6:4) would promote more effective DNA condensation.
To prepare these transfection complexes, DNA was added to a polylysine/lipid mixture, with the cationic liposomes in excess during vector assembly. Free liposomes were then removed by sucrose density gradient ultracentrifugation, leaving positively charged DNA/polylysine/lipid complexes with an optimal preparation ratio of 1/0.5/40 (w/w/m). The inclusion of polylysine in the lipid formulation is thought to promote greater DNA condensation due to its relatively higher charge density compared with cationic liposomes. Ultimately, higher transfection efficiencies were obtained using these vectors, when compared with cationic liposome formulations without polylysine, but only after purification and in the absence of serum. Furthermore, these positively charged particles were non-targetable as a result of non-specific association with negatively charged cell membranes. The authors coined the term liposome-entrapped polycation condensed plasmid DNA, or LPDI, to characterize these transfection complexes.

To avoid some of the limitations using LPDI vectors, Lee and Huang modified the LPDI formulation by changing both the preparation method and materials, and coined this new formulation LPDII [135,136]. This second strategy involves first condensing DNA with polylysine at a ratio of 4:3 (w/w) so that the positive charge is in moderate excess. These polylysine/DNA complexes, with a net positive charge, will then spontaneously associate with anionic liposomes. In contrast to the LPDI formulation, receptor targeting could be accomplished with LPDII vectors by controlling the lipid to DNA ratio to create complexes with a net negative charge (a schematic representation of LPDII formation is illustrated in Figure 1.8).
To test the transfection potential of the LPDII formulation, condensed plasmid DNA containing a luciferase reporter gene was combined with a pH-sensitive anionic lipid formulation consisting of DOPE/CHEMS/folate-PEG-DOPE (6:4:0.01 mol/mol), and administered to KB cells in vitro [136]. Transfection with LPDII vectors formulated with a low lipid:DNA ratio was independent of both folate receptor-targeting and pH-sensitivity. This observation can likely be explained by non-specific cellular uptake of transfection complexes containing a net positive charge. However, if the lipid:DNA ratio was increased to create negatively charged vectors, transfection efficiency and cellular uptake was folate-receptor mediated and required the presence of pH-sensitive lipids to promote liposomal and endosomal release of plasmid DNA into the cytoplasm. Moreover, these studies demonstrated that LPDII vectors mediated higher levels of transfection when compared with cationic liposome complexes, and were less cytotoxic.

In a subsequent report by Li and Huang, the strategy of introducing antisense oligonucleotides against the EGFR to promote inhibition of cell growth was combined with the LPDII formulation for gene delivery [137]. Results from these studies demonstrated that these approaches were complementary by observing downregulation of the EGFR after folate receptor-targeted LPDII oligonucleotide internalization in KB cells. Furthermore, delivery of antisense oligonucleotides was more efficient with LPDII compared with conventional PC/Chol/folate-PEG-DOPE liposomes.

Finally, recent studies by Reddy et al. demonstrated that the transfection efficiency of LPDII vectors can be enhanced by optimizing several components of the LPDII formulation [138]. First, a novel plasmid containing a 366-bp segment from SV40 DNA was employed to promote intracellular transport of the plasmid into the nucleus.
Moreover, several high molecular weight polymers, including acylated-polylysine and cationic dendrimers, were found to mediate more efficient condensation of plasmid DNA than unmodified polylysine, whereas smaller cationic molecules, such as spermine or gramicidin S, were less effective in this capacity. In addition, the pH-sensitive DOPE/CHEMS formulation was replaced with the ‘caged’ pH-sensitive N-Citraconyl-DOPE lipid formulation. When combined, these modifications promote greater transfection efficiency, while maintaining folate-mediated specificity and cellular uptake.

1.6.3 In vivo studies using folate receptor-targeted liposomes

In vivo data on folate receptor-targeted liposomes is noticeably absent from the literature. However, a recent study by Guo et al. evaluated the biodistribution of DSPC/Chol/PEG-DSPE/folate-PEG-Chol (60/34/5/1 m/m) liposomes containing 1 mol % DTPA-DSPE (a negatively charged lipid needed for radiolabeling with $^{111}$In) in C57BL/6 mice carrying syngeneic folate receptor-positive 24JK-FBP cell-derived subcutaneous tumor implants [125]. Folate-targeted liposomes carrying an $^{111}$In label were administered intravenously, and animals were sacrificed 24 h after dosing so tissues could be harvested for gamma counting (Figure 1.9). Not surprisingly, the organs accumulating the highest levels of radioactivity were the liver and spleen, which are known components of the RES. Furthermore, moderate accumulation of the radiolabel was observed in the kidney and in subcutaneous tumor implants. Unfortunately, the inclusion of folate for liposome targeting did not appear to significantly improve the overall level of liposome uptake in solid tumors.
1.7 Summary

Advances in liposome technology that permit extended residence times in the circulation, while reducing systemic toxicities, have led to useful formulations for the treatment of various malignancies. Moreover, improved methods for drug loading, and incorporation of genetic material in lipidic vectors, could extend the use of liposomal carriers for encapsulation of novel therapeutic agents or applications in gene therapy. *In vitro* studies evaluating the benefits of folate conjugation to liposomes provide encouraging results, suggesting that folate receptor-targeting has the potential to improve both the specificity and efficacy of liposomal therapeutics. However, these results must be confirmed *in vivo* to confirm the merit and clinical relevance of the various approaches discussed in this review.

Delineation of the exact folate internalization pathway will likely lead to improvements in folate-conjugated liposome formulations that can take advantage of the microenvironments to which folate receptor-targeted liposomes are exposed. It will likely be important to understand the normal folate pathway, in addition to realizing the effects of folate-conjugated liposomes on receptor disposition and internalization. Ultimately, obtaining a comprehensive understanding regarding the internalization of folate should allow for more logical vector development.

While folate receptor-targeting offers several advantages for the site-directed delivery of therapeutic agents to cells overexpressing the high affinity folate receptor, other targeting strategies that employ alternative low molecular weight ligands or antibodies may be useful in treating tumors with low folate receptor expression. In fact, significant
efforts have been made to develop successful antibody-targeted liposomes, with some success using anti-HER2 immunoliposomes among others [139,140].

Finally, gene therapy has the potential to revolutionize the treatments and outcomes of various genetic diseases. However, in order for this approach to succeed, vectors with the ability to deliver nucleic acids efficiently and specifically must be developed. Good progress towards this end has been achieved, but the ideal gene therapy vector is yet to be discovered. Folate receptor-targeted liposomes have the potential to be optimized for this purpose, and may prove invaluable in these treatment strategies.
1.8 Hypothesis and dissertation overview

Gene therapy has the potential to achieve significant improvements over existing treatments for genetic diseases, including cancer. However, there are several barriers that must be overcome before a beneficial therapeutic application of gene therapy is achieved. Many of these barriers are important for all gene therapy applications, but they are particularly relevant for the intravenous administration of non-viral transfection vectors for the treatment of cancer. Therefore, the rational design of vectors intended for this purpose should be developed with several factors in mind.

It is first necessary to create vectors that maintain stability in their original dosage form. Colloidal stability is important during storage, and ultimately manufacturing, to insure that vector integrity and function are preserved prior to administration. Some factors that affect colloidal stability include ζ-potential, charge distribution, particle size, surface hydrophilicity, concentration, and ionic strength. Second, if transfection vectors are administered by the intravenous route, they must be stable in the systemic circulation and avoid rapid clearance by components of the reticuloendothelial system. Plasma contains endo- and exo-nucleases that can cleave nucleic acids if introduced into the blood circulation unprotected. In addition, plasma also contains opsonins, albumin and lipoproteins that may bind to transfection vectors and alter their size and surface properties. Third, the size of vectors designed for intravenous administration should be minimized to promote extravasation through the fenestrated endothelium of the tumor vasculature. Small particles, less than 250 nm in diameter, could theoretically accumulate in solid tumors due the enhanced permeability and retention effect. This effect results from the increased permeability of the tumor vasculature, in combination...
with the poor lymphatic drainage in solid tumors. Fourth, vectors should be selectively internalized in target cells, while avoiding normal cells. Internalization and selectivity can theoretically be accomplished by introducing a tissue-specific targeting ligand, such as folic acid. Fifth, since DNA must ultimately be taken up by the nucleus for transcription, vectors for gene therapy should possess the ability to effectively escape endosomal compartments and promote delivery of DNA to the nucleus. Nuclear delivery of DNA should result in high levels of gene expression, which is currently the primary factor limiting the application of non-viral gene therapy. Finally, vectors should have low cellular and systemic toxicity, and avoid stimulation of the immune system.

Many vectors designed for gene therapy overcome one or more of these barriers, however, none currently possess all of the characteristics outlined for an effective gene delivery vehicle. This dissertation attempts to address the challenging problem of designing an improved transfection vector congruent with the criteria detailed above. The unifying hypothesis is that gene expression can be increased after non-viral transfection by introducing strategies that increase vector stability, improve endosomal release, and alter the internal characteristics and surface properties of vectors to enhance cellular uptake and gene expression. This hypothesis was tested by measuring the \textit{in vitro} expression of a luciferase reporter gene in various cell lines after transfection with polyethylenimine (PEI)-based non-viral vectors that incorporate reversible cross-linking reagents, pH-sensitive liposomes, polyethylene glycol (PEG), folate targeting-ligands, or a combination of these strategies.

In chapter two, low molecular weight PEI (800 Da) was cross-linked with the amine-reactive reducible cross-linking reagents dithiobis[succinimidylpropionate] (DSP) and
dimethyl 3,3'-dithiobispropionimidate 2 HCl (DTBP) to create relatively high molecular weight PEI for the formulation of polymer/DNA complexes (polyplexes). The hypothesis was that polyplexes prepared with cross-linked PEI would take advantage of the high transfection activity observed using high molecular weight PEI for DNA condensation and the reduced toxicity of low molecular weight PEI after transfection. In theory, the high molecular weight cross-linked PEI would mediate complete condensation of plasmid DNA prior to cellular uptake. However, once internalized and exposed to the reducing environment of the cytoplasm, the reducible disulfide bonds present in the cross-linking reagents would be reduced, thus promoting reversion of the cross-linked high molecular weight PEI to its low molecular weight counterpart. In addition to achieving lower toxicity, it was further speculated that disrupting the polymer structure might reduce the association between PEI and DNA, therefore, allowing easier access by the transcription machinery for gene expression.

To evaluate this strategy, PEI was cross-linked with either DSP or DTBP at different molar cross-linking ratios. The molecular weights of the resulting polymers were then estimated using calculations based on the inherent viscosity of PEI polymer solutions. The effects of cross-linking on gene expression were analyzed by measuring luciferase reporter gene activity after transfection of CHO cells in vitro. Toxicity of the vectors was assessed by measuring the amount of total cellular protein from cells remaining in each well after transfection. It was assumed that low protein levels were indicative of fewer adherent cells, and, therefore, greater toxicity. Finally, the reducibility of the disulfide bonds introduced by the cross-linking reagents was evaluated by agarose gel electrophoresis.
In chapter three, the development of a novel diolein-based pH-sensitive anionic liposome formulation for incorporation into gene delivery vectors is described. These liposomes were developed for the purpose of creating liposome-entrapped polycation-condensed DNA (LPD) vectors. LPDII vectors utilize polycations, such as PEI, to condense DNA into small nanometric particles, which are then associated with anionic pH-sensitive liposomes via electrostatic interactions. The hypothesis was that the gene transfer efficiency of LPDII vectors would be improved in the presence of serum by changing the lipid composition of the anionic liposomes used for LPDII formulation.

Previous LPDII formulations, which incorporate DOPE-based anionic liposomes, mediate significant levels of gene expression in vitro, as discussed earlier in this chapter. However, in the presence of serum, DOPE-based LPDII vectors lose their transfection activity. The rationale behind the experiments described in chapter three was to take advantage of the successful gene delivery using the LPDII vector design, but improve it by creating a formulation that maintained its transfection activity in the presence of serum. If non-viral vectors are ultimately to be administered by the intravenous route, serum-resistance is a prerequisite.

To create this new liposome formulation, diolein was combined with cholesteryl hemisuccinate (CHEMS), an anionic titratable cholesterol derivative. Initially, diolein and CHEMS were combined at various molar ratios, and the pH-sensitivity and transfection potential of each ratio was evaluated by particle size analysis and luciferase reporter gene expression, respectively. PEI with a molecular weight of 25 kDa was used as the DNA condensing agent in these studies. It was determined that diolein/CHEMS liposomes prepared at a molar ratio of 6:4 displayed favorable characteristics for
subsequent incorporation into LPDII vectors and should be further characterized. To confirm the pH-sensitivity of diolein/CHEMS liposomes, a calcein dequenching assay, based on the fluorescent properties of calcein, and particle size analysis was performed. The transfection activity of LPDII vectors prepared with diolein/CHEMS liposomes was then evaluated as a function of the PEI nitrogen/DNA phosphate (N/P) ratio and the lipid/DNA (L/D) weight ratio in vitro. To determine the contribution of the DNA condensing agent on transfection activity, diolein-based LPDII vectors were prepared using poly-L-lysine (PLL) instead of PEI. Gene expression was similarly evaluated as a function of L/D ratio for these PLL containing LPDII vectors. Next, transfection studies were performed in media containing up to 50% serum to evaluate whether luciferase gene expression was decreased under these conditions. Finally, diolein-based LPDII vectors were compared with other common transfection reagents, and transfection activity was analyzed in various cell lines.

In chapter four, diolein-based LPDII vectors were modified by incorporating DSP or DTBP cross-linked polyplexes, lipid anchored PEG, or PEG with a terminal folate moiety to mediate folate receptor-targeting. The hypothesis was that LPDII vector stability would be increased through cross-linking, and vector internalization into cells would be enhanced by incorporating a folate targeting-ligand to promote receptor-mediated endocytosis.

PEI, with a molecular weight of 25 kDa, was used to condense plasmid DNA prior to covalent cross-linking. This reaction scheme could theoretically yield PEI/DNA polyplexes with a caged structure resistant to dissociation. Polyplexes were cross-linked with DSP or DTBP at various molar ratios, and the transfection activity of these cross-
linked polyplexes was evaluated in KB cells to determine the effect of cross-linking on luciferase gene expression. Next, the stability of uncross-linked and cross-linked polyplexes was evaluated by measuring ethidium bromide (EtBr) fluorescence after exposure of the polyplexes to polymethacrylic acid (PMAA), an organic polyanion. It was reasoned that polyanionic liposomes would have similar effects on polyplex integrity as PMAA. The reducibility of the disulfide bonds present in the cross-linking reagents was evaluated by exposing cross-linked polyplexes to dithioerythritol (DTE) prior to PMAA, followed by EtBr fluorescence analysis and agarose gel electrophoresis. The transfection activity of diolein-based LPDII vectors containing cross-linked polyplexes, PEG, or a folate targeting-ligand was analyzed by measuring luciferase gene expression in folate receptor-positive KB cells as a function of the L/D ratio. In addition, transfection studies were also carried out in the presence of free folate to determine the contribution of folate receptor-targeting. Finally, transmission electron micrographs were obtained to confirm the association between PEI/DNA complexes and diolein/CHEMS liposomes, and to estimate the size of LPDII vectors.
**Figure 1.1 Structure of folic acid.** The $\alpha$ and $\gamma$ carboxyl groups of the glutamic acid moiety are labeled.
Figure 1.2 Multilamellar versus unilamellar liposomes. This illustration represents a schematic diagram of a single liposome in cross-section highlighting the differences between multilamellar and unilamellar liposomal vesicles.
Figure 1.3 Drug localization in a sterically stabilized folate receptor-targeted liposome. Hydrophilic and amphipathic molecules are incorporated in the aqueous interior inside liposomes, whereas lipophilic molecules are associated with the hydrocarbon chains of the phospholipid membrane. Also shown is a schematic illustration depicting folate conjugation for receptor-targeting, and steric stabilization of the liposome formulation by incorporation of the hydrophilic moiety poly(ethylene glycol) (PEG) on the liposome surface. PEG is thought to function by inhibiting incorporation of opsonins of the complement system, thus preventing recognition and clearance by the reticuloendothelial system.
Figure 1.4 Illustration of the L\(_\alpha\) bilayer phase (top) and type two inverted hexagonal phase (H\(_{II}\)) (bottom). At physiological pH dioleoylphosphatidylethanolamine (DOPE) will assume the H\(_{II}\) conformation. However, in the presence of negatively charged lipids, DOPE will assume the well-known L\(_\alpha\) bilayer conformation. This physical property allows formulation of pH-sensitive liposomes using DOPE. (adapted from [112] with permission)
Figure 1.5 Chemical structures of Folate-PEG-Chol and Folate-PEG-DSPE. Cholesterol (Chol) or distearoylphosphatidylethanolamine (DSPE) are molecules that can be incorporated in the liposome bilayer to serve as anchors for folate conjugation. As indicated in the figure, Chol and DSPE are separated from folate by a poly(ethylene glycol) (PEG) spacer, commonly 250 Å in length (M_r ~ 3350).

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Figure 1.6 Effect of folate derivatization on liposome uptake by cultured KB cells. KB cell monolayers were incubated with calcein-containing liposomes (60 nm) for 4 h at 37 °C and viewed by either phase contrast (left panels) or fluorescence microscopy (right panels). The upper two panels show the same field of cells following treatment with PEG liposomes lacking folate (control), whereas the lower two panels display a field of cells treated with folate-PEG liposomes. The absence of cell-associated calcein fluorescence in the control sample in contrast to the intense cell-associated fluorescence in the sample containing folate-derivatized liposomes reveals the sharp dependence of liposome recognition and uptake upon folate conjugation. Magnification is x 400. (adapted from [123] with permission)
Figure 1.7 Selective killing of HeLa cells in HeLa/W138 cell co-cultures.
HeLa/W138 cell co-cultures were treated for 1 h with 10 μM doxorubicin encapsulated in either folate-PEG-liposomes or non-targeted liposomes and examined under phase contrast microscope after 72 h further incubation. An untreated control culture is displayed in the left set of panels for comparison. (adapted from [126] with permission)
Figure 1.8 Possible mechanism for the formation of LPDII. The targeting ligand in this example is folate. (adapted from [136] with permission)
Figure 1.9 Biodistribution of folate-PEG-Chol targeted liposomes in tumor bearing mice. C57BL/6 mice (n = 3) bearing subcutaneous 24JK-FBP cell-derived tumors were treated with $^{111}$In-labeled liposomes. The mice were sacrificed 24 h after intravenous injection. (adapted from [125] with permission)
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CHAPTER 2

EFFICIENT GENE TRANSFER USING REVERSIBLY CROSS-LINKED LOW MOLECULAR WEIGHT POLYETHYLENIMINE

2.1 Abstract

Polyethylenimine (PEI) is a polycation with potential application as a non-viral vector for gene delivery. Here we show that subsequent to conjugation with homobifunctional amine reactive reducible cross-linking reagents, low molecular weight polyethylenimine efficiently mediates in vitro gene delivery to Chinese hamster ovary (CHO) cells. Two cross-linking reagents, dithiobis[succinimidylpropionate] (DSP) and dimethyl 3,3'-dithiobispropionimidate 2 HCl (DTBP), were utilized based on their reactivity and chemical properties. Both reagents react with primary amines to form reducible cross-links; however, unlike DSP, the DTBP cross-linker maintains net polymer charge through amidine bond formation. PEI with a reported weight-average molecular weight ($\bar{M}_w$) of 800 Da was reacted with either DSP or DTBP at PEI primary amine:cross-link reactive group ratios of 1:1 and 2:1. The transfection efficiencies of the resulting cross-linked products were evaluated in CHO cells using a luciferase reporter gene under a cytomegalovirus (CMV) promoter. Our results show that cross-linked polymers mediate variable levels of transfection depending on the cross-linking reagent, extent of
conjugation, and the N/P ratio. In general, we found conjugate size to be proportional to gene transfer efficiency. Using gel retardation analysis, we also evaluate the capacity of the cross-linked polymers to condense plasmid DNA before and after reduction with 45 mM dithiothreitol (DTT). DTT mediated reduction of intracross-link disulfide bonds and inhibited condensation of DNA by conjugates cross-linked with DSP at a ratio of 1:1, but had little effect on the remaining polymers. Analogous intracellular reduction of transfection complexes by reduced glutathione could facilitate uncoupling of PEI from DNA to enhance gene expression.
2.2 Introduction

The primary obstacle towards implementing an effective gene therapy using non-viral vectors remains their relatively inefficient gene delivery in vivo. Although viral vectors are able to overcome this problem, and generally yield higher gene expression, there are concerns over the immunogenic, cytotoxic and recombinogenic potential of viral gene transfer. Due to the improved safety profile and ease of preparation and manipulation, non-viral techniques for gene delivery continue to be explored and optimized.

Polyethylenimine (PEI) is a cationic polymer which has proven to be an effective transfection agent both in vitro (1-3) and in vivo (4-8). Due to the chemical properties of PEI, primarily the high cationic charge density, it is able to effectively condense DNA and form nanometric particles capable of being endocytosed (9). In addition, since every third atom in the polymer is an amino nitrogen capable of being protonated, PEI effectively buffers the endosomal environment, thus protecting DNA from lysosomal nuclease degradation and facilitating endosomal escape to the cytoplasm. Cellular uptake of PEI-DNA complexes likely results from nonspecific electrostatic interactions between positively charged polymer-DNA complexes and negatively charged residues on the cell surface (10). Several groups have demonstrated improved transfection efficiencies with PEI by incorporating targeting ligands such as galactose (11), mannose (12), transferrin (13) or antibodies (14,15).

Dithiobis[succinimidylpropionate] (DSP) is a homobifunctional NHS (N-hydroxysuccinimide) ester-based electrophilic cross-linking reagent containing an 8 atom spacer 12 Å in length (16). Dimethyl 3,3'-dithiobispropionimidate 2 HCl (DTBP) is a similar molecule containing an 8 atom spacer 11.9 Å in length, but utilizes an imidoester...
reactive group to form amidine bonds resulting in the retention of net positive charge (16). Both cross-linking reagents react with primary amines to form stable covalent linkages, and are constructed around a centrally located disulfide bond, which is cleavable with common reducing agents after conjugation (Figure 2.1).

It is well established that branched high molecular weight polyethylenimines (>25 kDa) are effective transfection agents, and recent reports indicate that relatively low molecular weight PEI (~10 kDa) can also be used to deliver DNA efficiently with lower toxicity (17,18). With this in mind, we hypothesized that high molecular weight conjugates composed of cross-linked 800 Da PEI would yield transfection efficiencies comparable to those obtained with high molecular weight PEI. Furthermore, we expected that when the transfection complexes were exposed to the reducing environment of the cytoplasm that the disulfide bonds introduced by the cross-linking reagents would be cleaved. This reduction would promote reversion of the high molecular weight complexes back to their low molecular weight counterparts, presumably leading to lower toxicity and potentially easier access by the transcription machinery (Figure 2.2).

In this report, we examine the transfection properties of low molecular weight PEI cross-linked with either DSP or DTBP in CHO cells. We also evaluate the DNA condensing properties of the cross-linked conjugates before and after reduction with 45 mM DTT as a means to determine disulfide-bond reducibility, and therefore reversibility of cross-linking, after conjugation.
2.3 Materials and Methods

2.3.1 Polymers, Plasmid and Cross-linking Reagents

PEI with $M_\text{w}$ of 800 Da and 25 kDa were purchased from Aldrich, and used as 0.16 mM aqueous solutions (3 nmol N/µl) in all transfection experiments. Plasmid DNA containing the luciferase reporter gene, pcDNA3-CMV-Luc (7.1 kb), was kindly provided by Dr. Leaf Huang (University of Pittsburgh Medical Center). Cloning and preparation of plasmid DNA were performed by propagating transformed E. Coli in LB Medium containing ampicillin (50 µg/mL), followed by isolation and purification with a commercially available plasmid purification kit (Qiagen). Concentration and purity of DNA were assessed spectrophotometrically by measuring absorbance at wavelengths of 260 nm and 280 nm ($OD_{260}/OD_{280} \sim 1.7$). Plasmid size and integrity were confirmed using agarose gel electrophoresis (0.9% agarose gel containing 0.5 µg/mL ethidium bromide) and cleavage with the restriction endonuclease Hind III (Gibco BRL). Cross-linking reagents, dithiobis[succinimidylpropionate] (DSP) (MW-404.42) and dimethyl 3,3'-dithiobispropionimidate 2 HCl (DTBP) (MW-309.28), were purchased from Pierce.

2.3.2 PEI Cross-linking with DSP and DTBP

Prior to cross-linking, PEI (800 Da) was labeled with fluorescein isothiocyanate (FITC) (Sigma) at a molar ratio of 100:1 (PEI:FITC) to aid in concentration determination of the cross-linked polymers. Briefly, 800 mg PEI was dissolved in 4 mL of DMSO for a final concentration of 200 mg/mL. Next, 389.4 µL of FITC dissolved in DMSO (10 mg/mL) was added dropwise to the PEI solution while vortexing. The
reaction was allowed to proceed in the dark at room temperature (RT) for 30 min, and was then stored at 4°C protected from light. Precipitation of the labeled polymer confirmed that the reaction between PEI and FITC proceeds to completion, shown by the absence of free FITC in the supernatant. PEI-FITC was then cross-linked with either DSP or DTBP at PEI primary amine:cross-link reactive group ratios of 1:1 and 2:1. Based on reports in the literature (17,19) we estimated the primary amine content of PEI (800 Da) to be 25% of the total amines. To achieve PEI primary amine:cross-link reactive group molar ratios of 1:1 or 2:1, 117 mg or 58.5 mg of the appropriate cross-linking reagent was dissolved in 1 mL or 0.5 mL DMSO, respectively. The DSP or DTBP solutions were then added dropwise to PEI-FITC solutions containing 100 mg PEI-FITC in approximately 550 µL DMSO. The reactions were then allowed to proceed for 1 h at RT. Prior to use, aliquots of cross-linked polymer samples were diluted 1:4 with double distilled (dd) H₂O to a volume of 0.5 mL. The diluted samples were then purified on a 1.5 x 8 cm Sephadex® G-25 fine column using ddH₂O as the eluent under atmospheric pressure. One mL fractions were collected, and the concentration of each fraction was determined spectrophotometrically by measuring absorbance at 495 nm and extrapolating concentration from a standard curve obtained with uncross-linked PEI-FITC of known concentrations. Finally, each sample was diluted to 0.16 mM (3 nmol N/µL) for use in transfection experiments.

2.3.3 Transfection of CHO Cells

CHO cells were cultured in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), 100 units/mL penicillin, 100 µg/mL streptomycin,
300 μg/mL L-glutamine and 100 μM sodium citrate (Gibco BRL). For transfection experiments, 1.5 x 10^5 cells/well were seeded on 24-well plates (Falcon) and allowed to incubate for approximately 20 h in normal growth media at 37°C in a humidified atmosphere containing 5% CO₂ (~70% confluent). Prior to transfection, the media were replaced with 0.75 mL serum-free growth medium. Polymer-DNA complexes were prepared in 37.5 mM NaCl solutions by addition of 150 μL PEI or cross-linked PEI-FITC to 7.5 μg pcDNA3-CMV-Luc plasmid DNA to achieve the desired N/P ratios in a final volume of 300 μL (experiments performed in triplicate for 100 μL/well or 2.5 μg DNA/well). Following a 20 min incubation at RT, the transfection complexes were applied to CHO cells and allowed to incubate for 4 h. The media was then replaced with normal growth media supplemented with 10% FBS, and luciferase gene expression was measured 24 h later using a commercially available kit (Promega). Protein concentrations were determined using the bicinchoninic acid (BCA) protein determination assay (Pierce), with bovine serum albumin (BSA) standards (Sigma).

2.3.4 Gel Retardation Assays

An aliquot of 1 μg pcDNA3-CMV-Luc plasmid DNA was condensed with PEI or cross-linked PEI at selected N/P ratios by addition of polymer to DNA in 37.5 mM NaCl solution. Prior to electrophoresis (0.9% agarose gel containing 0.5 μg/mL ethidium bromide) the complexes were incubated at RT for 20 min. To evaluate the effect of a reducing agent on DNA condensation, some complexes were incubated further for 30 min in the presence of 45 mM DTT (Sigma). An aliquot of 0.5 μg of DNA were loaded in each well.
2.3.5 Viscosity Measurements

A 1 mL aliquot of PEI or cross-linked PEI was prepared at a concentration (c) of 50 mg/mL in ddH₂O. The time required for each sample to flow through a 1 mm (outside diameter) capillary tube under atmospheric pressure was recorded, and these values were used to calculate the inherent viscosity (\( \eta_{\text{inh}} \)) of the polymer solutions. The \( \eta_{\text{inh}} = \ln \eta_{\text{rel}} / c \), where \( \eta_{\text{rel}} \) is the relative viscosity which equals \( t_{\text{solution}} / t_{\text{solvent}} \), or the time required for the polymer-solvent solution to flow through the capillary tube divided by the time required for the solvent alone. The molecular weight of cross-linked polymers was estimated by comparison with unconjugated polyethylenimine standards.
2.4 Results

2.4.1 Transfection efficiency of various polyethylenimines in CHO cells at selected N/P ratios

CHO cells were transfected *in vitro* with 2.5 μg of pcDNA3-CMV-Luc plasmid DNA condensed with various polyethylenimines. Luciferase gene expression was quantified as relative light units (RLU) and fmol luciferase standardized for protein concentration and corrected for background (Figure 2.3). The data show that among the different PEI polymers tested, 800 Da PEI was the least effective transfection agent. In fact, gene expression using this vector was immeasurable below an N/P ratio of 18. This finding is consistent with previous reports, which show negligible gene expression for PEI with nominal molecular weights below 1,800 Da (1). In contrast, cells transfected with 25 kDa PEI produced high levels of luciferase activity, yielding a peak luciferase expression level of $2 \times 10^9$ RLU/mg protein at a N/P ratio of 9.

Transfection with cross-linked 800 Da PEI-FITC yielded a variety results depending on the cross-linking reagent, the extent of conjugation and the N/P ratio. The DTBP conjugate prepared at a PEI primary amine:imidoester reactive group ratio of 2:1 (DTBP 2:1) was relatively ineffective, yielding gene expression on the order of $10^4$-$10^6$ RLU/mg protein. Changing the cross-linking ratio to 1:1 (DTBP 1:1) increased gene expression by several orders of magnitude at N/P ratios ≥ 9. Interestingly, no luciferase activity was detected using this conjugate at a N/P ratio of 5. This result was surprising given the fact that the DTBP 2:1 conjugate was able to mediate gene transfer at this ratio, albeit at relatively low levels. The DSP conjugate prepared at a PEI primary amine:NHS reactive group ratio of 2:1 (DSP 2:1) yielded gene expression over a wide range, $10^3$-$10^8$ RLU/mg
protein, facilitating higher gene expression at larger N/P ratios. Analogous to the DTBP conjugates, we found that increasing the cross-linking ratio to 1:1 (DSP 1:1) led to increased luciferase activity. In fact, the DSP 1:1 conjugate was able to mediate the highest levels of transgene expression among the cross-linked polymers tested, $4 \times 10^8$ RLU/mg protein at N/P ratios of 9 and 13.

2.4.2 Protein concentrations from cellular lysates after transfection with various polyethylenimines

By measuring protein concentrations of cellular lysates from adherent cells in each well after transfection we were able to infer the relative toxicities of cross-linked polymers in relation to unmodified PEI (Figure 2.4). We assumed an inverse relationship between the protein concentration and cytotoxicity, reasoning that lower protein concentrations were indicative of fewer adherent cells and therefore greater toxicity. Wells transfected with 25 kDa PEI consistently produced the lowest protein concentrations, yielding less protein as the charge ratio increased. This finding corroborated earlier reports of toxicity observed after transfection with high molecular weight PEI at large N/P ratios (3). Conversely, protein concentrations measured from cells transfected with 800 Da PEI remained steady at approximately 175 µg/mL at all charge ratios, and yielded the highest protein concentrations among the transfection agents studied.

Similar to the gene expression data, conjugated PEI-FITC yielded intermediate protein concentrations depending on the cross-linking reagent, degree of conjugation and the N/P ratio. PEI-FITC conjugated with DSP at a ratio of 1:1 produced the lowest
protein concentrations among cross-linked polymers. Analogous to the trend observed with 25 kDa PEI, concentrations decreased with increasing charge ratio. The DTBP 1:1 conjugate yielded a similar profile, but protein values were higher than those found with DSP 1:1 and 25 kDa PEI. Conjugates prepared at a ratio of 2:1 yielded concentrations ≥ 125 µg/mL, and at higher charge ratios did not display the characteristic decrease observed with the 1:1 conjugates.

2.4.3 Gel retardation analysis of PEI-FITC cross-linked with DSP at a primary amine: NHS reactive group ratio of 1:1

Using gel retardation analysis, we evaluated the DNA condensing properties of the various polyethylenimines used for transfection before and after reduction with 45 mM DTT. We hypothesized that reduction of intramolecular disulfide bonds present in the cross-linked PEI would inhibit DNA condensation by disrupting the structure of the high molecular weight conjugates. However, we found this to be true only in the case of DSP conjugates formed at a ratio of 1:1 (Figure 2.5). Our results showed that at an N/P ratio of 5, the DSP 1:1 conjugate was unable to completely condense DNA as shown by the lack of ethidium bromide exclusion. However, after DTT reduction, this effect was even more pronounced and very little DNA condensation was observed. In contrast, at an N/P ratio of 9, DNA was visualized only after cleavage with DTT. At higher charge ratios, condensation was complete regardless of disulfide bond integrity. Unmodified PEI, both the 800 Da and 25 kDa, effectively condensed DNA at all charge ratios examined (data not shown). This result is in agreement with previous work, which showed complete DNA condensation with PEI above N/P ratios of 2 (11).
2.4.4 Polymer molecular weights calculated from inherent viscosity

Finally, using inherent viscosity calculations we were able to approximate the weight-average molecular weights of the cross-linked PEI conjugates (Table 2.1). We found that 800 Da PEI cross-linked with DTBP at a PEI primary amine:imidoester reactive group ratio of 2:1 yielded conjugates with an average molecular weight of nearly 8 kDa. Increasing the cross-linking ratio to 1:1 led to an increase in the molecular weight, yielding conjugates around 23 kDa. Similarly, PEI cross-linked with DSP at a PEI primary amine:NHS ester reactive group ratio of 2:1 also produced conjugates with an average molecular weight of about 23 kDa. The DSP 1:1 conjugate yielded the largest molecular weight, with a value of approximately 75 kDa. These results showed that DSP mediated more extensive cross-linking at equal cross-linking ratios.
2.5 Discussion

For non-viral vectors to be clinically useful, it will be necessary to develop formulations that mediate high levels of cell specific gene expression with favorable toxicity profiles. Here we built on the foundation of successful gene delivery using polyethylenimine by evaluating a new strategy to enhance gene expression. Previous research has shown that several parameters affect the performance of polyethylenimine vectors for gene delivery (3, 20-23). Our findings verified the importance of polymer molecular weight on transfection efficiency. We showed that cross-linking low molecular weight PEI with homobifunctional amine-reactive cross-linking reagents to form high molecular weight conjugates led to efficient transfection of CHO cells in vitro. In general, transfection results showed that DSP conjugates outperformed DTBP conjugates at similar cross-linking ratios. Furthermore, although 25 kDa PEI proved to be the most effective transfection agent, cross-linked PEI-FITC achieved gene expression of similar magnitude at higher N/P ratios.

Based on relative viscosity measurements, we obtained approximate weight-average molecular weights for PEI-FITC conjugated with DSP and DTBP. Cross-linking 800 Da PEI-FITC with DTBP at a ratio of 2:1 produced conjugates with an average molecular weight of nearly 8 kDa. Therefore, it was not surprising to find that this conjugate consistently yielded relatively low gene expression. The DTBP 1:1 and DSP 2:1 conjugates had similar molecular weights around 23 kDa. However, transfection experiments showed that these polymers mediated variable degrees of gene expression despite their similar size. Specifically, the DSP 2:1 conjugate proved less effective at lower N/P ratios. Finally, cross-linking PEI with DSP at a ratio of 1:1 led to the
formation of conjugates having an approximate molecular weight of 75 kDa. As expected, this conjugate yielded the highest luciferase activity among the cross-linked polymers tested.

The success of the cross-linking reactions, characteristics of the resulting conjugates and subsequent transfection performance could be explained, in part, by the chemical nature of the cross-linking reagents utilized for conjugation. At identical cross-linking ratios, we found DTBP conjugates to have lower molecular weights than DSP conjugates. We suspect that this difference was due to electrostatic repulsion between the positive charge carried on the imidoester reactive group of DTBP and the highly cationic polyethylenimine polymer. This repulsion likely led to less extensive cross-linking, and ultimately smaller conjugates. Differences in the inherent reactivities of the cross-linking reagents towards primary amimes could also have played a role. Although DSP mediated more extensive cross-linking, reaction of the DSP NHS esters with PEI primary amines led to the elimination of positive charges carried by the polymer, whereas amidine bonds introduced by DTBP compensated for this loss. In effect, DTBP conjugates were able to achieve higher gene expression than DSP conjugates of similar molecular weight at equal N/P ratios because even though the N/P ratios were the same, the charge ratios were different. The increase in net charge for DTBP conjugates would promote greater interaction between positively charged transfection complexes and negatively charged cell membranes, potentially leading to increased gene transfer.

Measurements of protein concentration allowed us to infer the relative toxicities of the various transfection complexes. In general, wells transfected with DSP conjugates yielded lower protein concentrations (higher toxicity) than DTBP conjugates formed at
analogous cross-linking ratios, although this effect was more pronounced with conjugates prepared at a ratio of 1:1. Furthermore, protein levels decreased to a greater extent at high N/P ratios with conjugates cross-linked at a ratio of 1:1. The lack of increased toxicity at higher charge ratios was particularly encouraging for the DSP 2:1 conjugate, which showed relatively high gene expression at a N/P ratio of 18. Furthermore, although 25 kDa PEI yielded higher transfection it also produced the greatest toxicity among the polymers tested.

To our knowledge, this is the first report describing the transfection properties of cross-linked low molecular weight polyethylenimine. However, cross-linking strategies have been used previously to increase the transfection performance and stability of non-viral vectors. Trubetskoy et al. utilized DTBP to cross-link the amine groups of polylysine and polyallylamine after condensation of DNA to form a caged DNA complex that increased their stability in salt solution (24). McKenzie et al. hypothesized that incorporating cysteine residues into low molecular weight peptides would result in the formation of reducible disulfide bridges between the cysteine residues after DNA condensation. They found they were able to create small, stable complexes with enhanced transfection properties in several cell lines (25). Our work showed that 800 Da PEI-FITC cross-linked with DSP or DTBP efficiently transfected CHO cells in vitro with reduced overall toxicity. Furthermore, in the case of conjugates cross-linked with DSP at a ratio of 1:1, reduction of disulfide bonds decreased the DNA condensing properties of this polymer. Since the cytoplasmic environment is markedly reducing, it is reasonable to expect that disulfide bonds introduced via cross-linking reagents will be reduced intracellularly by reduced glutathione, leading to the breakdown of PEI conjugates. In
fact, glutathione reductase acts essentially in one direction so that the ratio of reduced glutathione to oxidized glutathione is approximately 500:1 in most cells (26). Although transfection efficiency using conjugated polymers did not appear to be superior to 25 kDa PEI, there was the apparent advantage of reduced toxicity. Transfection with polyethylenimine could lead to an accumulation of PEI localized in the nucleus. This PEI would be able to interact with endogenous DNA, potentially leading to undesirable side effects. Using cross-linked polymers at the appropriate conjugation ratio would alleviate these concerns due to a decrease in the affinity for DNA observed after reduction of intracross-link disulfide bonds.

In closing, cross-linking low molecular weight PEI with DSP or DTBP may confer a pharmacokinetic advantage to polyethylenimine vectors in vivo. PEI is an organic polymer that cannot be metabolized or broken down by cellular enzymes. As a result, total body clearance of high molecular weight PEI will likely be a slow process, potentially leading to an accumulation of the polymer in vivo. The introduction of disulfide bonds through DSP and DTBP should promote reversion of the high molecular weight transfection complexes to their low molecular weight counterparts. These low molecular weight fragments should be cleared more easily from the body due to enhanced diffusion and potentially easier filtration through glomerular capillaries.
Table 2.1  Approximate weight average molecular weights of cross-linked low molecular weight polyethylenimine. Polyethylenimine (PEI) was cross-linked with either DTBP or DSP at PEI primary amine:cross-link reactive group ratios of 2:1 or 1:1. Using inherent viscosity, the approximate weight average molecular weights of cross-linked polymers was calculated.
Figure 2.1 Proposed reaction scheme for conjugating polyethylenimine with crosslinking reagents DSP and DTBP. Also shown is the effect of disulfide bond reduction by DTT.
Figure 2.2 Proposed transfection scheme using reversibly cross-linked low molecular weight polyethylenimine. Following endocytosis and subsequent endosome release, intracellular reduction of polymer disulfide bonds leads to the release of DNA for nuclear uptake and transcription.
Figure 2.3 Transfection efficiency of various polyethylenimines in CHO cells at selected N/P ratios. Results are shown as relative light units (RLU)/mg protein on the left ordinate, and fmol luciferase/mg protein on the right ordinate. Transfection agents are identified in the figure legend.
Figure 2.4 Protein concentrations from cellular lysates after transfection with various polyethylenimines. Solid circles - 800 Da PEI, open circles - 25 kDa PEI, solid triangles - PEI-FITC cross-linked with DTBP at a primary amine:imidoester reactive group ratio of 2:1, open triangles - PEI-FITC cross-linked with DTBP at a primary amine:imidoester reactive group ratio of 1:1, solid squares - PEI-FITC cross-linked with DSP at a primary amine:NHS reactive group ratio of 2:1, open squares - PEI-FITC cross-linked with DSP at a primary amine:NHS reactive group ratio of 1:1.
Figure 2.5 Gel retardation analysis of PEI-FITC cross-linked with DSP at a primary amine: NHS reactive group ratio of 1:1. Lane 1 is phage λ DNA digested with Hind III used as a size marker; lane 2 is pcDNA3-CMV-Luc plasmid DNA only; lanes 3-5 are plasmid DNA condensed with the DSP 1:1 conjugate at N/P ratios of 5, 9 and 13, respectively; lanes 6-8 are identical to lanes 3-5 except the polymer-DNA complexes were treated with 45 mM DTT.
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CHAPTER 3

CHARACTERIZATION OF A NOVEL DIOLEIN-BASED LPDII VECTOR FOR GENE DELIVERY

3.1 Abstract

LPDII vectors are non-viral vehicles for gene delivery comprised of polycation-condensed plasmid DNA (polyplexes) complexed with anionic pH-sensitive liposomes. Here, we describe a novel LPDII formulation containing polyethylenimine (PEI) polyplexes complexed with anionic pH-sensitive liposomes composed of diolein/cholesteryl hemisuccinate (CHEMS) (6:4 mol/mol). The pH-sensitivity of diolein/CHEMS liposomes was evaluated through quantitative fluorescence measurements of calcein release and particle size analysis. The results indicated that diolein/CHEMS liposomes were stable at physiological pH, but underwent rapid aggregation and fluorescence dequenching at pH values ≤ 5.0. Using a luciferase reporter gene, in vitro transfection of KB oral cancer cells showed that the transfection efficiency of LPDII vectors was superior to other well-characterized polyplexes and lipoplexes. Results further showed that gene delivery using diolein-containing LPDII vectors was dependent on the PEI nitrogen/DNA phosphate (N/P) ratio, the lipid/DNA weight ratio and the cell line being...
transfected. Replacing PEI with poly-L-lysine as the DNA condensing agent resulted in a moderate reduction in transfection activity. Moreover, in contrast to LPDII formulations incorporating dioleoylphosphatidylethanolamine (DOPE), the transfection efficiency of diolein-based LPDII vectors was sustained in media containing up to 50% fetal bovine serum. Since diolein-based LPDII vectors mediated efficient gene transfer and retained their transfection activity in the presence of serum, diolein may be a promising alternative to DOPE for the construction of non-viral vectors for \textit{in vivo} gene delivery.
3.2 Introduction

Recently, significant efforts have been devoted to the development of safe and efficient vectors suitable for the systemic administration of gene therapy. Non-viral vectors, although generally not as efficient as viral vectors, are attractive because they are associated with fewer safety concerns and are easier to produce in clinically relevant quantities. In addition, they are potentially less immunogenic and have fewer restrictions on their capacity to carry DNA. Among non-viral vectors, lipoplexes are perhaps the best characterized [1,2]. Currently, however, their utility in gene therapy is hampered by: (i) toxicity and immunostimulatory activities of the cationic lipids used for lipoplex assembly, (ii) limited in vivo transfection efficiency, and (iii) low tissue specificity. These shortcomings may be due to inadequate DNA condensation, poor colloidal stability and incompatibility with the abundance of negatively charged macromolecules present in the physiological environment [3-7].

In an attempt to overcome these obstacles, LPDII transfection vectors, consisting of polycation-condensed DNA entrapped in anionic pH-sensitive liposomes, have been developed [8]. To formulate LPDII vectors, polyplexes are first prepared by condensing plasmid DNA with a cationic polymer to form nanometric particles containing a net positive charge. These polyplexes are then complexed to anionic pH-sensitive liposomes, via electrostatic interactions, to form LPDII vectors with either a net positive or net negative charge [8]. The capacity to formulate neutral or negatively charged transfection vectors is important, since these vectors are potentially more compatible with the physiological environment of the systemic circulation. Furthermore, neutral or
negatively charged vectors should be more amenable to tissue-specific delivery, after incorporation of a targeting ligand, by reducing non-specific electrostatic interactions with cells [8,9].

Poly-L-lysine (PLL) and polyethylenimine (PEI) have both been utilized in LPDII vectors as the DNA-condensing agent, with similar efficacy [10]. However, variability in the composition of the anionic liposomes used to make LPDII vectors drastically changes their transfection potential. For example, previously published results demonstrate that dioleoylphosphatidylethanolamine (DOPE)-based LPDII vectors mediate efficient LPDII gene transfer to cultured cells [8,10-14]. In contrast, when LPDII vectors are formulated with the non-fusogenic lipid dioleoylphosphatidylcholine (DOPC), a complete loss of transfection activity is observed [8].

Despite the success that has been attained using DOPE-containing LPDII vectors, their utility is currently limited by a loss of transfection efficiency in the presence of serum, thus presenting a major barrier toward their application as a systemic gene therapy vehicle. Therefore, in the present study, we describe the development and characterization of a novel “serum-resistant” LPDII formulation, containing di-9-octadecenoylglycerol, or diolein, as the fusogenic lipid component. The pH-sensitive properties of the diolein/CHEMS liposomes were characterized, followed by an evaluation of the in vitro gene transfer properties of diolein-based LPDII vectors. Transfection efficiency was analyzed with respect to polyplex charge ratio, lipid/DNA weight ratio, serum concentration and cell lineage. The results indicate that incorporation
of diolein into LPDII formulations could potentially enhance the usefulness of LPDII vectors as \textit{in vivo} carriers for gene therapy.
3.3 Materials and Methods

3.3.1 Materials

Poly-L-lysine (PLL, M₉ ~ 29,000), calcein, cholesteryl hemisuccinate (CHEMS),
dimethyldioctadecylammonium bromide (DDAB), 1,2 dioleoyloxypropyl-3-trimethyl-
ammonium chloride (DOTAP), and di-9-octadecenoyl-glycerol (diolein, ~ 85% 1,3- and
15% 1,2-isomer) were purchased from Sigma Chemical Company (St. Louis, MO).
Polyethylenimine (PEI, M₉ ~ 25,000) was purchased from Aldrich Chemical Co.
(Milwaukee, WI). Dioleoylphosphatidylethanolamine (DOPE) was purchased from
Avanti Polar Lipids (Alabaster, AL). Luciferase assay reagents were obtained from
Promega (Madison, WI). Polycarbonate membranes and the handheld LiposoFast
extruder were obtained from Avestin Inc. (Ottawa, ON). BCA protein assay reagents
were purchased from Pierce Chemical Co. (Rockford, IL). Tissue culture media and
supplies were purchased from Life Technologies (Rockville, MD).

3.3.2 Plasmid DNA preparation

pCMV-Luc plasmid DNA, encoding the firefly luciferase reporter gene under
control of the cytomegalovirus enhancer/promoter, was obtained as a gift from Dr.
Leaf Huang at the University of Pittsburgh. Plasmid DNA was isolated and
purified from DH5-α E. Coli using the Qiagen mega plasmid purification kit
(Qiagen, Santa Clarita, CA). DNA concentration and purity were quantified by UV
absorbance at 260 nm and 280 nm on a Shimadzu UV-160U Spectrophotometer.
The structural integrity and topology of purified DNA was analyzed by agarose gel electrophoresis.

3.3.3 Preparation of calcein-containing liposomes

Calcein-loaded liposomes, composed of diolein/CHEMS (6:4 mol/mol) or DOPE/CHEMS (6:4 mol/mol), were prepared by a polycarbonate membrane extrusion method, as described previously [15]. Briefly, a chloroform solution of the lipid mixture, containing 9 mg of total lipid, was dried as a thin film on the wall of a 13 x 100 mm glass tube under a stream of nitrogen gas. Residual chloroform was further removed by placing the tube in a dessicator under vacuum for 30 min. The lipid mixture was then hydrated with 1 mL of an 80 mM calcein solution prepared in phosphate-buffered saline (PBS: 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.5). The suspension was sonicated for 5 min in a bath sonicator (model 50HT, VWR Scientific Product, Inc.), subjected to 6 cycles of freezing and thawing, and then extruded 10 times through a 0.2 μm pore-size polycarbonate membrane, using a handheld LiposoFast extruder. Unentrapped calcein was separated from liposomes by gel filtration on a 10-mL Sepharose CL-4B column equilibrated in PBS. The final calcein concentration in liposome preparations was calculated from the absorbance at 495 nm, using a molar extinction coefficient of 80,000 cm⁻¹M⁻¹.
3.3.4 pH sensitivity of liposomes

The release of liposome contents in response to decreasing pH was determined using a calcein dequenching assay, as described previously [16]. Fluorescence measurements were performed on a Perkin-Elmer LS-50-B spectrofluorometer operated with an FTWinlab (Morena Valley, CA) computer program. The excitation and emission wavelengths were 495 nm and 520 nm, respectively, with a slit width of 5 nm for both. Calcein-loaded liposomes, containing 45 µg of lipid, were added to 2 mL of PBS (pH 7.5) or sodium acetate buffers at various pH (100 mM NaCl, 10 mM acetate, pH 5.0, 5.5, 6.0, and 6.5). After a 30 min incubation at 37 °C, calcein fluorescence was measured. Due to the slight effect of pH on the calcein absorption spectrum, the fluorescence intensities were adjusted for differences in fluorescence at acidic pH. The percentage of calcein release was defined as:

\[
\% \text{ Calcein Release} = \left(\frac{I_{\text{pH}} - I_o}{I_{100} - I_o}\right) \times 100 \%
\]

where \(I_o\) is the fluorescence at neutral pH, \(I_{100}\) is the fluorescence after the addition of 0.15% Triton X-100 at neutral pH, and \(I_{\text{pH}}\) is the fluorescence intensity at acidic pH before the addition of Triton X-100.

The time-course of liposome aggregation in response to buffer pH was also determined over a pH range of 4.8-7.4. Liposomes, containing 60 µg of lipid, were added to 400 µL of buffers of various pH at room temperature or 37 °C. The mean particle diameter and size distribution of the liposomes were measured at various times.
3.3.5 Preparation of LPDII vectors

Anionic liposomes, composed of diolein/CHEMS (6:4 mol/mol) or DOPE/CHEMS (6:4 mol/mol), were prepared by an ethanol injection method, as described previously [10]. Briefly, a chloroform solution of the lipid mixture, containing 3 mg of total lipid, was dried as a thin layer on the wall of a 13 x 100 mm glass tube under a stream of nitrogen gas. The lipids were then dried further in a dessicator under vacuum for 30 min. The lipid film was re-dissolved in 50 μL of ethanol, and was then quickly injected into 1 mL sterile HEPES buffer (20 mM, pH 8.0) under vortexing. The mean particle diameters for diolein and DOPE liposomes were 120 nm and 100 nm, respectively. The liposome samples were stored at 4°C, and were used for LPDII formation within 2 weeks of preparation.

To prepare LPDII particles, 1 μg plasmid DNA and the appropriate amount of polycation (PEI or PLL) needed to achieve a particular polycation nitrogen/DNA phosphate ratio were each diluted in 20 μL of serum-free RPMI 1640 growth media prior to mixing. After mixing, the solution was briefly vortexed, and the resulting polyplexes were incubated for 10 min at room temperature. Anionic liposomes, diluted in 40 μL of serum-free media, were then added to the polyplexes under mild vortexing to form LPDII particles.
3.3.6 Preparation of polyplexes and lipoplexes

Cationic liposomes, with the composition DOTAP/DOPE (1:1 mol/mol) or DDAB/DOPE (1:1 mol/mol), were prepared as described previously [10]. To prepare lipoplexes, 1 μg plasmid DNA and various amounts of cationic liposomes, each diluted in 20 μL of serum-free RPMI 1640 growth media, were mixed under vortexing. Similarly, PEI/DNA polyplexes were prepared by mixing 1 μg plasmid DNA with the desired amount of PEI, each diluted in 20 μl of serum-free RPMI 1640 growth media, to generate a PEI nitrogen/DNA phosphate (N/P) ratio of 10 [17]. The resulting lipoplexes or polyplexes were incubated for 10 min at room temperature before use.

3.3.7 Cell culture and transfection procedures

KB human oral cancer cells were obtained as a gift from Dr. Philip Low at Purdue University (West Lafayette, IN). 24JK-FBP, a methylcholanthrene-induced mouse sarcoma cell line retrovirally transfected with the human folate receptor-α gene, was obtained as a gift from Dr. Patrick Hwu at the National Cancer Institute. Both cell lines were cultured in RPMI 1640 growth media supplemented with 10% fetal bovine serum (FBS). B16, a mouse melanoma cell line, F98, a human glioma cell line, and CHO, Chinese hamster ovary cells, were obtained as gifts from Dr. Rolf Barth at the Ohio State University College of Medicine. CHO cells were maintained in F12 nutrient media, while B16 and F98 cells were maintained in DMEM growth media, all supplemented with 10% FBS. Cells were cultured continuously as monolayers at 37 °C in a humidified atmosphere containing 5% CO₂.
For transfection studies, ~10^5 cells/well were seeded on 24-well plates 24 h prior to transfection, achieving 70-80% confluence. Unless otherwise specified, all transfection experiments were carried out in standard growth media containing 10% FBS, and were performed in triplicate. Cells were incubated with transfection vectors, containing 1 μg plasmid DNA, in 500 μL of culture media for 4 h at 37 °C. Alternatively, preliminary transfection studies were performed by incubating cells with transfection complexes for 2 h. The transfection medium was then removed, and the cells were incubated for a further 24 h in fresh culture medium before analysis of luciferase activity.

To assay luciferase gene expression, cells were rinsed 3 times with PBS prior to cell lysis (lysis buffer: 0.5 % Triton X-100, 100 mM Tris-HCl, 2 mM EDTA, pH 7.8). The cellular lysates were then centrifuged at 13,000 RPM for 2 min, and the supernatants analyzed for protein content, using the BCA protein determination assay, and luciferase activity, using a commercially available kit. Relative light units (RLU) were measured with a Mini-Lum luminometer (Bioscan, Inc., Washington DC), and were converted to pg luciferase using a standard curve, generated under identical assay conditions, using recombinant luciferase standards.
3.4  Results

3.4.1  Determination of the optimal lipid ratio for diolein/CHEMS liposomes

To determine the optimal lipid ratio of diolein/CHEMS liposomes, preliminary stability and pH-sensitivity studies were performed with five liposome formulations prepared at a series of different molar ratios, namely 5:5, 6:4, 7:3, 8:2 and 9:1. Diolein/CHEMS liposomes prepared at molar ratios beyond 8:2 yielded large lipid aggregates, which persisted after extensive vortexing and sonication, and were, therefore, excluded from further evaluation. Preliminary stability results for the remaining three formulations, shown in Figure 3.1A, indicated that diolein/CHEMS liposomes prepared at molar ratios of 5:5, 6:4 and 7:3 all exhibited sufficient colloidal stability at physiological pH, and similar sensitivity to acid-induced aggregation at a pH of 4.2. However, subsequent analysis of LPDII mediated transfection activity in KB oral cancer cells, displayed in Figure 3.1B, showed that LPDII vectors formulated with diolein/CHEMS liposomes, containing a PEI nitrogen/DNA phosphate (N/P) ratio of 4 and lipid/DNA (L/D, w/w) ratio of 5, prepared at molar ratios of 5:5 and 6:4 yielded >14-fold and 33-fold higher luciferase activities, respectively, compared with LPDII vectors formulated with liposomes prepared at a molar ratio of 7:3. Furthermore, LPDII vectors incorporating liposomes prepared at a molar ratio of 6:4 yielded a luciferase activity >2-fold higher than that achieved with liposomes prepared at a molar ratio of 5:5. The 6:4 diolein/CHEMS lipid ratio was, therefore, selected for further characterization and incorporation into LPDII vectors in subsequent studies.
3.4.2 Acid-induced destabilization of diolein/CHEMS liposomes

Since the transfection activity of LPDII vectors is largely determined by the fusogenic properties of their lipid component, studies were performed to characterize the pH-sensitivity of diolein/CHEMS (6:4) liposomes using a calcein dequenching assay and particle size analysis. Liposomes were prepared by polycarbonate membrane extrusion in the presence of 80 mM calcein, a concentration that generates complete self-quenching. An encapsulation efficiency of 2.6 % was obtained, which is consistent with passive entrapment of calcein. The liposomes were then incubated in buffers of variable pH for 30 min at 37 °C, and analyzed by fluorometry for the degree of calcein dequenching. As shown in Figure 3.2, the percentage of calcein released from diolein/CHEMS liposomes increased as the buffer pH decreased. At pH 5.0, 40 % of the encapsulated calcein was released from diolein/CHEMS liposomes. In contrast, at the same pH, control liposomes composed of DOPE/CHEMS (6:4), a well-characterized pH-sensitive liposome formulation, released 67 % of their contents.

To further assess the effects of pH on diolein/CHEMS liposomes, particle size analysis was performed to determine the extent and rate of liposome aggregation induced by acidic pH, both at RT and at 37 °C. The results, shown in Figures 3.3A and 3.3B, reveal that liposome stability at physiological pH and aggregation at acidic pH were relatively independent of temperature, under the imposed experimental conditions. The mean diameter of diolein/CHEMS liposomes, after a 60-min incubation at a pH of 4.8, increased from approximately 150 nm to
approximately 1700 nm and 1500 nm at RT and 37 °C, respectively. In contrast, very little change in particle size was observed for liposomes incubated at pH 7.4 and pH 5.35 for the same duration. Furthermore, at pH 4.8, particle size appeared to increase in a near linear fashion beyond a 10 min incubation period.

3.4.3 Transfection activity of diolein-containing LPDII vectors

To evaluate gene transfer efficiency, diolein/CHEMS (6:4) liposomes were combined with PEI-condensed plasmid DNA, carrying the firefly luciferase reporter gene, to form LPDII vectors with a series of different L/D (w/w) and N/P ratios. The transfection activities of the various LPDII formulations were then evaluated in human KB cells, an oral carcinoma cell line. As shown in Figure 3.4A, the transfection activity of LPDII vectors was dependent on both the N/P and L/D ratios. Luciferase gene expression initially increased with increasing L/D ratio, reaching a maximum between 1 and 2.5, before decreasing slowly with further increases in the L/D ratio. Transfection activity was less dependent on polyplex N/P ratio, at ratios ≥ 3, although larger N/P ratios did appear to mediate higher luciferase activities at very low L/D ratios (≤ 0.25). At an N/P ratio of 2, condensation of plasmid DNA is known to be incomplete, and, therefore minimal transfection was observed, regardless of the L/D ratio.

To evaluate the role of the cationic polymer on transfection efficiency, LPDII vectors were also generated using poly-L-lysine (PLL) as the DNA-condensing agent. Because the ε-amino groups on lysine residues (pKₐ > 10) carry a permanent
charge at physiological pH, a PLL nitrogen/DNA phosphate ratio of 2 provided sufficient positive charge for DNA condensation and formation of LPDII vectors. Transfection of KB cells using PLL containing LPDII vectors showed only a modest reduction in reporter gene expression, compared to vectors incorporating PEI, as shown in Figure 3.4B. Since PLL has been shown to have minimal endosomal lytic activity, the observed transfection activity using PLL-containing LPDII vectors was likely due to the fusogenic property of the diolein/CHEMS liposomes [18-20].

3.4.4 The effect of serum on LPDII transfection efficiency

As shown in Figure 3.5, the presence of 10% FBS in the transfection media had a favorable effect on the transfection efficiency of diolein-containing LPDII vectors in KB cells, increasing luciferase gene expression by more than 2-fold compared to cells transfected in serum-free media. Significantly, further increases in the FBS concentration, up to 50%, did not result in a decrease in the transfection efficiency of diolein-containing LPDII vectors.

3.4.5 Comparison of the transfection activity among non-viral vectors

To assess the relative transfection efficiency of diolein-containing LPDII vectors, gene expression was compared against several non-viral formulations, including PEI polyplexes, two different lipoplex formulations and LPDII vectors.
prepared with DOPE/CHEMS liposomes (6:4 mol/mol). Lipoplexes were prepared using two well-characterized cationic liposome formulations, namely DDAB/DOPE (1:1 mol/mol) and DOTAP/DOPE (1:1 mol/mol). As shown in Figure 3.6, diolein-containing LPDII vectors produced > 10-fold higher levels of luciferase activity compared to either lipoplex formulation. Similarly, PEI polyplexes, in the absence of pH-sensitive liposomes, also mediated lower transfection activity than diolein-containing LPDII vectors, suggesting that the lipid component in LPDII vectors promoted more efficient gene delivery. Furthermore, Figure 3.6 also shows that transfection levels mediated by LPDII vectors formulated with DOPE/CHEMS liposomes were highly sensitive to the presence of serum, yielding luciferase levels orders of magnitude lower than those observed using DOPE-based LPDII vectors in serum-free media, and for diolein-based LPDII vectors.

3.4.6 LPDII transfection efficiency in various cell lines

The transfection activity of LPDII vectors was further assessed in a number of different mammalian cell lines, including B16, a murine melanoma, 24JK, a sarcoma cell line, F98, a human glioma cell line, and CHO, Chinese hamster ovary cells. As observed with other non-viral vectors, the transfection efficiency of diolein-containing LPDII vectors was variable depending on the cell line being transfected, as shown in Figure 3.7. With the exception of F98 cells, which exhibited luciferase expression of ~ 1.5 x 10^3 pg luciferase/mg protein, transfection levels using diolein-based LPDII vectors were on the order of 10^1 pg luciferase/mg.
protein. Among the cell lines tested, KB cells consistently displayed the highest level of luciferase activity. Furthermore, based on visual inspection and quantitative measurements of protein concentration in cellular lysates after transfection, all cell lines appeared to tolerate LPDII-mediated transfection well.
3.5 Discussion

Successful implementation of gene therapy depends on the development of gene transfer vectors that are safe, efficient, and tissue specific. The theory behind the LPDII design is to increase transfection efficiency by combining the DNA condensing properties of cationic polymers with the endosomal lytic activity of fusogenic pH-sensitive liposomes. In fact, this design is reminiscent of the structure of an enveloped virus particle. Previous studies showed that LPDII vectors mediate higher gene transfer efficiency and exhibit greater colloidal stability compared to some well-characterized lipoplexes and polyplexes [8]. Furthermore, LPDII vectors do not involve the use of cationic lipids, therefore potentially avoiding toxicity resulting from non-specific stimulation of cytokine production [9].

Since endosomal escape of the DNA contained in LPDII vectors is likely mediated by the fusogenic properties of the lipid component, the anionic liposome composition has a profound impact on the transfection properties of LPDII vectors. All previously reported LPDII formulations incorporated DOPE, or some derivative, as the pH-sensitive fusogenic lipid component [8,10-12]. DOPE is a cone-shaped molecule favoring membrane phase transition from a bilayer to a hexagonal II phase. In DOPE-containing liposomes, stability at neutral pH and pH-sensitivity are provided by the incorporation of an anionic amphiphile with a titratable headgroup, such as CHEMS, oleic acid, or citraconyl-DOPE [10-12]. The resulting liposomes are stable at neutral pH, but become fusogenic in response to
the low pH found in endosomal compartments, thus providing a mechanism for endosomal disruption and cytosolic escape. A critical defect in pH-sensitive liposome formulations containing DOPE is that these liposomes tend to lose their fusogenic properties in the presence of serum, possibly due to modification of the bilayer composition by protein insertion and/or lipid extraction [21]. As a result, LPDII formulations containing DOPE are ineffective in the presence of serum, as shown in Figure 3.6, thus preventing their utility for systemic gene delivery. Therefore, these studies were performed to characterize an alternative fusogenic lipid for LPDII assembly that remains active in the presence of serum, namely diolein.

Diolein, a diacylglycerol (DAG), has a cone-shape geometry similar to that of DOPE [22]. The polar portion of diolein, consisting of a single hydroxyl moiety, occupies a minimal hydrodynamic volume, which may be further reduced by its ability to form a hydrogen bond with the headgroup of an adjacent diolein molecule, as depicted in Figure 3.8, or an oxygen atom on a nearby phospholipid molecule. These structural features predict a high propensity for diolein to promote the transition of lipids from a bilayer phase to an inverted hexagonal II phase, facilitating membrane fusion. In fact, Takahashi et al. previously reported that diolein induces structural perturbations in phospholipid bilayers [23]. Moreover, the presence of diolein in pure phospholipid membranes causes a marked decrease in the transition temperature from lamellar to hexagonal II phase [24,25], and treatment of liposomes with phospholipase C causes vesicle fusion, a process mediated by the formation of diolein [24,25]. In the present study, calcein-
containing diolein/CHEMS liposomes showed pH-dependent calcein leakage and irreversible particle size increases at low pH, similar to liposomes composed of DOPE/CHEMS. Although these results are consistent with membrane fusion in response to acidic pH, further studies are required to elucidate the mechanism of gene delivery mediated by diolein-containing LPDII vectors.

Branched PEI, with a molecular weight of 25 kDa, was used as the DNA condensing agent for LPDII formation in these studies. Based on published data, it is conceivable that PEI may improve gene delivery by facilitating nuclear localization of internalized DNA molecules following endosomal release [26]. However, the reported endosomal lytic activity of PEI is unlikely a main contributor to the transfection activity exhibited by the LPDII vectors characterized in this study. This is based on the fact that PLL, which has little endosomal lytic activity of its own [18-20], also produced significant luciferase activity when chosen as the DNA condensing agent for LPDII formation.

In contrast to LPDII particles prepared with DOPE/CHEMS liposomes, which are inactivated by the presence of serum, LPDII particles containing diolein/CHEMS liposomes mediated efficient gene transfer when transfection was carried out in the presence of serum. This retention of transfection activity was presumably due to the differences between diolein and DOPE in their interactions with serum components. Alternatively, diolein-based LPDII vectors may have a tendency to aggregate in serum containing media, thereby increasing particle size. An increase in particle size would promote sedimentation of the transfection
complexes onto the cell monolayer, thus leading to increased transfection levels, as has been shown for polyplex mediated gene delivery [27]. Moreover, an increase in particle size could also explain the relatively high gene expression observed using diolein-based LPDII vectors formulated with a net negative charge, which, otherwise, would theoretically lead to lower transfection levels due to reduced interactions with the negatively charged cell membrane, in the absence of a targeting ligand to promote receptor-mediated vector internalization. Another possible explanation for the high transfection activity mediated by negatively charged transfection vectors is that LPDII particle assembly may be saturable beyond a certain threshold, although the transfection results in figure 3.4A reveal a gradual decline in transfection activity as the lipid/DNA ratio is increased, thus making this possibility somewhat less likely.

In light of the observed success using transfection vectors containing diolein, diolein may also find utility as a fusogenic lipidic component in other types of drug delivery vehicles, such as drug-carrying pH-sensitive liposomes or cationic liposomes. Furthermore, any safety concerns associated with the clinical use of diolein as gene/drug carriers should be minimal, since diolein is present endogenously in humans as a product of triglyceride metabolism [28]. In fact, ~ 1-15% of our dietary intake of oils and fats consists of diolein [29]. Moreover, diolein is a component of plant-based oils and fats, and is approved by the Food and Drug Administration for use as a component of emulsifiers [28].
3.6 Conclusion

In summary, a novel LPDII formulation was developed utilizing diolein as the endosomal lytic component. This non-viral formulation demonstrated significant transfection activity in the presence of serum, and transfection was well tolerated by cultured cells. Future modification of LPDII vectors through incorporation of polyethylene glycol conjugated lipids, to increase the circulation half-life, or targeting ligands, to enhance tissue-specific internalization, should be feasible through modification of the anionic liposome formulation utilized for LPDII vector assembly. In fact, work is currently in progress to characterize the effects these modifications may have on LPDII mediated transfection efficiency. Due to the promising in vitro transfection profile, further studies are warranted to explore the potential applications of diolein-based LPDII vectors for systemic delivery of gene therapy constructs.
Figure 3.1 Stability of diolein/CHEMS liposomes and transfection efficiency of diolein-based LPDII vectors using various diolein/CHEMS liposome formulations. (A) Mean diameter of diolein/CHEMS liposomes, prepared at molar ratios of 5:5, 6:4 and 7:3, evaluated over a 30 min time interval at pHs 7.4 and 4.2 (B) Transfection efficiency of LPDII vectors prepared at an N/P ratio of 4 and L/D ratio (w/w) of 5, containing diolein/CHEMS liposomes prepared at molar ratios of 5:5, 6:4 and 7:3. LPDII vectors contained 1 μg plasmid DNA, and transfections were carried out for 2 h on KB oral cancer cells in normal growth media containing 10% FBS. Data are presented as mean ± s.d. (n=3).
Figure 3.1 continued...
Figure 3.2 Acid-induced calcein leakage from liposomes. Liposomes, composed of diolein/CHEMS (6:4 mol/mol) and DOPE/CHEMS (6:4 mol/mol), were incubated for 30 min at 37 °C in PBS (pH 7.5) or sodium acetate buffers of different pH. The fluorescence intensity was measured before and after addition of 0.15 % Triton X-100. Percentage of calcein release was calculated as described in Materials and Methods. Data are presented as mean ± s.d. (n=3).
Figure 3.3 Time dependent effects of pH on the mean diameter of diolein/CHEMS liposomes. Diolein/CHEMS liposomes were incubated with buffers of various pH at (A) room temperature or (B) 37 °C, and liposome mean particle diameter was then measured at the indicated time points. Data are presented as mean ± s.d. (n=3).
Figure 3.4 Transfection of KB cells with LPDII vectors prepared with diolein/CHEMS liposomes. (A) Transfection efficiency of LPDII vectors prepared at various PEI nitrogen/DNA phosphate and lipid/DNA (L/D) ratios. (B) Transfection efficiency of LPDII vectors containing polylysine (N/P of 2) at various L/D ratios. LPDII vectors were incubated with KB cells in normal growth media containing 10% FBS for 4 h at 37 °C. The transfection medium was then replaced with fresh growth media, and luciferase activity was measured 24 h later. Data are presented as mean ± s.d. (n=3).
Figure 3.4 continued...

![Graph showing the relationship between Lipid/DNA Ratio (w/w) and Luciferase/mg Protein. The graph displays a curve that peaks at a lipid/dna ratio of approximately 5.0 and then decreases as the ratio increases.]
Figure 3.5 Influence of serum concentration on the transfection activity of LPDII vectors prepared with diolein/CHEMS liposomes. The vectors were prepared at a PEI nitrogen/DNA phosphate ratio of 4, and a lipid/DNA weight ratio of 2.5. Transfection was carried out by incubating KB cells with transfection vectors for 4 h at 37 °C, in the presence of various concentrations of FBS (v/v). Data are presented as mean ± s.d. (n=3).
Figure 3.6 Comparison of LPDII, PEI, and cationic liposome-mediated transfection.
KB cells were transfected with 1 μg plasmid DNA prepared in various formulations. (A) PEI polyplexes, at a PEI nitrogen/DNA phosphate (N/P) ratio of 10. (B) DDAB/DOPE (1:1 mol/mol) lipoplexes, lipid/DNA (L/D) weight ratio of 5. (C) DOTAP/DOPE (1:1 mol/mol) lipoplexes, L/D weight ratio of 5. (D) LPDII vectors (composed of DOPE/CHEMS, 6:4 mol/mol) with an N/P ratio of 4 and an L/D ratio of 5, under serum free conditions. (E) LPDII vectors (composed of DOPE/CHEMS, 6:4 mol/mol) with an N/P ratio of 4 and an L/D ratio of 5 (F) LPDII vectors (composed of diolein/CHEMS, 6:4 mol/mol) at an N/P ratio of 4 and an L/D ratio of 2.5. All transfections were carried out in the presence of 10% FBS, except where noted. Data are presented as mean ± s.d. (n=3).
Figure 3.7 Transfection efficiency of LPDII vectors in various cell lines. LPDII vectors were formulated with diolein/CHEMS liposomes and PEI-condensed DNA, prepared at an N/P ratio of 4 and a lipid/DNA weight ratio of 2.5. Data are presented as mean ± s.d. (n=3).
Figure 3.8 Proposed structure of intermolecular hydrogen bonding between diolein molecules. Six molecules of diolein are drawn in the schematic diagram. Approximately 85% of the molecules are represented as the 1,3-isomer, with the remaining 15% represented as the 1,2-isomer, so as to reflect the composition of the diolein used in liposome preparation.
References


4.1 Abstract

LPDII vectors are synthetic vehicles for gene delivery composed of polycation condensed DNA complexed with anionic liposomes. In this study, we evaluated the stability and transfection properties of polyethylenimine (PEI, 25 kDa)/DNA polyplexes before and after covalent cross-linking with dithiobis(succinimidylpropionate) (DSP) or dimethyl 3,3'-dithiobispropionimidate 2HCl (DTBP), either alone or as a component of LPDII vectors. We found that cross-linking PEI/DNA polyplexes at molar ratios ≥ 10:1 (DSP or DTBP:PEI) stabilized these complexes against polyanion disruption, and that this effect was reversible by reduction with 20 mM dithioerythritol (DTE). Transfection studies with polyplexes cross-linked at molar ratios of 10:1-100:1 in KB cells, a folate receptor-positive oral carcinoma cell line, showed decreasing luciferase gene expression with increasing cross-linking ratio. Subsequently, polyplexes, cross-linked with DSP at a molar ratio of 10:1, were combined with anionic liposomes composed of diolein/cholesteryl hemisuccinate (CHEMS) (6:4 mol/mol), diolein/CHEMS/polyethylene glycol-distearoylphosphatidylethanolamine (PEG-DSPE)
(6:4:0.05 mol/mol) or diolein/CHEMS/folate-PEG-cholesterol (folate-PEG-Chol)
(6:4:0.05 mol/mol) for LPDII formation. Transfection studies in KB cells showed that
LPDII vectors containing cross-linked polyplexes mediated ~ 2-15-fold lower gene
expression than LPDII prepared with uncross-linked polyplexes, depending on the
lipid:DNA ratio. Inclusion of PEG-DSPE at 0.5 mol % appeared to further decrease
transfection levels ~2-5-fold. Compared with LPDII formulated with PEG-DSPE,
LPDII incorporating 0.5 mol % folate-PEG-Chol exhibited higher luciferase activities at
all lipid:DNA ratios tested, achieving an ~ 10-fold increase at a lipid:DNA ratio of 5.
Compared with cross-linked LPDII vectors without PEG-DSPE, inclusion of folate-PEG-
Chol increased luciferase activities 3-4-fold between lipid:DNA ratios of 1-5.
Interestingly, inclusion of 1 mM free folate in the growth media during transfection
increased transfection activity ~ 3-4-fold for cross-linked LPDII vectors and LPDII
containing folate-PEG-Chol, but had no effect on the transfection activity of LPDII
formulated with PEG-DSPE. However, in the presence of 5 mM free folate, the
luciferase activity mediated by LPDII vectors containing folate-PEG-Chol was reduced ~
6-fold. Transmission electron micrographs were also obtained to provide evidence of
LPDII complex formation. Cross-linked LPDII vectors appeared as roughly spherical
aggregated complexes with a rather broad size distribution ranging between 300-800 nm.
4.2 Introduction

Non-viral delivery of gene therapy is an emerging strategy for the treatment of genetic disease, but progress is currently limited by inadequacies in the vectors available for gene transfer (1). The main deficiency of non-viral vectors is their relatively low in vivo gene transfer efficiency, compared with viral vectors. Therefore, research efforts have been focused on the design of non-viral vectors capable of achieving high tissue-specific gene expression with low immunogenicity and minimal toxicity. One particular strategy being evaluated to achieve these goals involves the use of LPD (liposome-entrapped polycation-condensed DNA) vectors containing covalently cross-linked polyplexes for gene delivery.

LPD vectors are synthetic vehicles for gene delivery composed of polycation condensed DNA (polyplex) complexed with cationic or anionic liposomes via electrostatic interactions. Early LPD formulations, coined LPDI, utilized poly-L-lysine (PLL) condensed DNA in association with cationic liposomes composed of dioleoylphosphatidylethanolamine/1-β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DOPE/DC-Chol; 6:4 mol/mol) (2). Subsequent alteration of the lipid component led to the development of LPDII transfection vectors. In contrast to LPDI, LPDII vectors are prepared with anionic pH-sensitive liposomes, such as dioleoylphosphatidylethanolamine/cholesteryl hemisuccinate (DOPE/CHEMS; 6:4 mol/mol) (3, 4). Since LPDII can be prepared with a net negative charge, these vectors have greater potential for receptor targeting, due to reduced non-specific electrostatic interactions with non-target cells. Moreover, neutral or negatively charged transfection vectors should be more compatible with the physiological environment of the systemic
circulation, due to reduced interactions with components of the blood and decreased activation of the complement system (5).

First generation LPDII vectors, those formulated with DOPE/CHEMS liposomes, exhibit nearly a complete loss of transfection activity in the presence of serum. Recently, our laboratory developed a method to circumvent this limitation through incorporation of a novel anionic pH-sensitive liposome formulation composed of diolein/CHEMS (6:4 mol/mol) (Guo et al., in press). Transfection studies with LPDII vectors containing diolein/CHEMS liposomes showed that transfection activity was sustained even when transfection was carried out in the presence of 50 % fetal bovine serum (Guo et al., in press). In addition, the DNA condensing agent can consist of polycations other than PLL, such as polyethylenimine (PEI) (6) or cationic dendrimers (7), without negatively affecting gene expression.

Recent efforts in our laboratory have been directed toward assessing the utility of homobifunctional cross-linking reagents for the development of reducibly cross-linked polyplexes for gene delivery (8). Two cross-linking reagents, dithiobis(succinimidyl-propionate) (DSP) and dimethyl 3,3'-dithiobispropionimidate 2HCl (DTBP) (9), were utilized to create relatively high molecular weight polymers, composed of cross-linked low molecular weight PEI, for polyplex assembly. DSP is a homobifunctional N-hydroxysuccinimide (NHS) ester-based electrophilic cross-linking reagent containing an 8-atom spacer 12 Å in length. DTBP is similar to DSP in structure and length, but contains an imidoester reactive group for amidine bond formation. The significance of the amidine bond is that net molecular charge is retained after conjugation with DTBP, whereas amide bond formation mediated by DSP results in the elimination of two
positive charges. Both cross-linking reagents react with primary amines to form stable
covalent bonds, and are constructed around a centrally located disulfide linkage that is
cleavable with common reducing agents after conjugation (Figure 4.1).

In the present study, we describe a novel LPDII transfection vector containing a
covalently stabilized PEI-based polyplex core complexed with anionic pH-sensitive
liposomes composed of diolein/CHEMS. We hypothesized that since LPDII formation is
predicated on electrostatic interactions between a cationic polymer and plasmid DNA
initially, and subsequently between cationic polyplexes and anionic liposomes, that
covalent cross-linking of the polyplex core, after DNA condensation, would stabilize the
polyplex against dissociation due to charge competition from the anionic lipid
component. Therefore, studies were performed to assess the effects of cross-linking
PEI/DNA polyplexes with DSP or DTBP on polyplex stability and transfection potential,
and subsequently the transfection performance of LPDII vectors containing covalently
cross-linked polyplexes in folate receptor-positive KB oral cancer cells in vitro.
Furthermore, additional studies were performed to evaluate the effect of introducing a
lipid-anchored folate receptor-targeting ligand or hydrophilic polyethylene glycol (PEG)
coating into the LPDII formulation. Covalent attachment of folate for receptor targeting
has proven to be effective in vitro for targeting liposomes (10-12) and LPDII vectors (3)
to cancer cells which overexpress the high affinity folate receptor. In addition,
incorporation of PEG into liposome formulations enhances tumor accumulation in vivo
by reducing clearance by the reticuloendothelial system (RES), thus extending the
circulation half-life (13, 14).
4.3 Materials and Methods

4.3.1 Materials

Cholesteryl hemisuccinate (CHEMS), di-9-octadecenoyl-glycerol (diolein, ~ 85 % 1,3- and 15 % 1,2-isomer), dithioerythritol (DTE), bovine serum albumin (BSA) protein standards, ethidium bromide (EtBr) and other general use chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Dithiobis(succinimidylpropionate) (DSP), dimethyl 3,3'-dithiobispropanoimidate 2HCl (DTBP) and bicinchoninic acid (BCA) protein assay reagents were purchased from Pierce (Rockford, IL). Anhydrous dimethyl sulfoxide (DMSO), polyethyleneimine (PEI, branched, Mr ~ 25,000) and polymethacrylic acid (PMAA, Mw ~ 6,500) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Luciferase assay reagents and recombinant luciferase were obtained from Promega (Madison, WI). All tissue culture media and supplies were purchased from Gibco BRL (Rockville, MD).

4.3.2 Plasmid preparation

pcDNA3-CMV-Luc (pLuc, 7.1 kb) plasmid DNA encoding the firefly luciferase gene under control of the cytomegalovirus enhancer/promoter was obtained as a gift from Dr. Leaf Huang at the University of Pittsburgh School of Pharmacy. Cloning and preparation of plasmid DNA was performed by propagating transformed DH5-α E. Coli in LB media containing 50 μg/mL ampicillin, followed by isolation and purification with a commercially available plasmid purification kit (Qiagen, Valencia, CA). Concentration and purity of DNA were assessed spectrophotometrically by measuring absorbance at 260 and 280 nm (OD\textsubscript{260}/OD\textsubscript{280} ~ 1.7). Plasmid size and integrity were confirmed using
agarose gel electrophoresis (0.9% agarose gel containing 0.5 µg/mL ethidium bromide) and cleavage with the restriction endonuclease Hind III (Gibco BRL).

4.3.3 Cell culture

KB cells, a folate receptor-positive human oral squamous cell carcinoma, were obtained as a gift from Dr. Philip Low at Purdue University. The cells were cultured in folate-free RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin and 300 µg/mL L-glutamine at 37 °C in a 5% CO₂ humidified atmosphere. FBS provided the only source of folate in the growth media, which achieved physiologically relevant folate concentrations.

4.3.4 Liposome preparation

Three pH-sensitive anionic liposome formulations were prepared: diolein/CHEMS (6:4 mol/mol); diolein/CHEMS/PEG (M₉ ~ 2,000)-distearoylphosphatidylethanolamine (DSPE) (6:4:0.05 mol/mol); and diolein/CHEMS/folate-PEG (M₉ ~ 3,350)-Cholesterol (Chol) (6:4:0.05 mol/mol). PEG-DSPE was purchased from Avanti Polar Lipids, Inc., and folate-PEG-Chol was synthesized as described previously (15). Liposomes were prepared by mixing ~8-15 mg of total lipid (dissolved in chloroform as 20 mg/mL solutions) at the appropriate ratios in 13 x 100 mm glass test tubes. The chloroform was evaporated under a stream of nitrogen, and the lipids were further dried in a dessicator under vacuum for 30 min. The resulting lipid film was rehydrated with 20 mM HEPES buffer to a concentration of 3.33 mg/mL, and adjusted to pH 8.0-8.5. The suspensions were then vortexed and sonicated in a bath sonicator until the mean particle diameter was
between 100-150 nm, as determined by dynamic light scattering using a NICOMP submicron particle sizer Model 370. Lipid suspensions were stored at 4 °C until use.

4.3.5 Preparation of PEI/DNA polyplexes and stabilization by cross-linking

For polyplex preparation, 10 µg pLuc DNA and the amount of PEI required to achieve the desired N/P (PEI nitrogen:DNA phosphate) ratios were each diluted in 250 µL HEPES buffer (20 mM, pH 8.0). The PEI solution was quickly added to the DNA, and the resulting solution was then mixed and allowed to stand at room temperature (RT) for 15 min. For covalent cross-linking of polyplexes, DSP or DTBP, dissolved in anhydrous DMSO as a concentrated stock solution (1-10 mg/mL), was added to polyplexes at various concentrations depending on the desired cross-linking ratio. Cross-linking ratios are expressed as DSP:PEI molar ratios, and DMSO was kept below 5% of the total volume of polyplex solutions. After addition of the appropriate cross-linking reagent, samples were incubated at RT for 1 and 3 hours for DSP and DTBP, respectively. Preparation of cross-linked and uncross-linked polyplexes for transfection studies was performed similarly, with exceptions noted below.

4.3.6 Stability of cross-linked polyplexes against polyanion disruption with PMAA

Polyplex stability against polyanion disruption was examined by measuring the ability of polymethacrylic acid (PMAA) to restore DNA access to ethidium bromide (EtBr) binding. Polyplexes with various N/P ratios containing 10 µg pLuc DNA in 500 µL HEPES buffer (20 mM, pH 8.0), either with or without DSP cross-linking, were diluted into 1.9 mL 0.1X phosphate-buffered saline (PBS: 136.9 mM NaCl, 2.68 mM
KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4). PMAA was added at various concentrations and incubated with polyplexes for 30 min at RT. To evaluate the reducibility of the disulfide bond present in the cross-linking reagent, 20 mM dithioerythritol (DTE) was added to some samples prior to the PMAA incubation. EtBr was present in the solution at a final concentration of 0.4 µg/mL, and EtBr fluorescence was measured on a Perkin-Elmer LS-50-B spectrometer, operated with a FTWinlab computer program (Morena Valley, CA). The excitation and emission wavelengths were set at 523 nm, with a 5 nm slit width, and 602 nm, with a 10 nm slit width, respectively.

Polyplex stability was further evaluated by agarose gel electrophoresis with EtBr staining. Polyplexes containing 1 µg pLuc DNA prepared at a N/P ratio of 6, either with or without DSP cross-linking, were incubated with 100 µM PMAA for 30 min at RT. The reducibility of cross-linking was analyzed by including 20 mM DTE during the incubation. Half of each sample (0.5 µg DNA) was then loaded on a 0.9 % agarose gel containing 0.5 µg/mL EtBr, and run at 120 V for ~ 90 min.

4.3.7 Transfection of KB cells with cross-linked polyplexes or LPDII vectors

For transfections, 10⁵ KB cells/well were seeded in 24-well plates (Falcon) and allowed to incubate in folate-free RPMI 1640 growth media containing 10 % FBS for approximately 20 h at 37 °C in a 5 % CO₂ humidified atmosphere (~ 70-80 % confluence). Immediately prior to transfection, cells were rinsed with warm PBS, and 0.5 mL folate-free RPMI 1640 growth media was added to each well. LPDII transfections were carried out in the presence of 10 % FBS, whereas polyplex transfections were performed under serum-free conditions. Transfection complexes were added to each well.
and incubated with KB cells for 2 or 4 h for LPDII vectors or polyplexes, respectively. After rinsing the cells with 300 μL warm PBS, the transfection media was replaced with 0.5 mL fresh RPMI containing 10% FBS. A further 24 h incubation was imposed, followed by cell lysis and analysis of luciferase gene expression (see below).

PEI/pLuc polyplexes (N/P = 10) utilized for transfection were prepared as described above for EtBr fluorescence analysis, but with 1 μg DNA and the desired amount of PEI, each dissolved in 30 μL 20 mM HEPES buffer prior to mixing. Various concentrations of DSP or DTBP, dissolved in DMSO, were then added to polyplex solutions to achieve different cross-linking ratios, and incubated at RT for 1 or 3 h, respectively. DMSO comprised less than 5% of the solution volume.

LPDII vectors, containing PEI/pLuc polyplexes (N/P = 4) and diolein/CHEMS liposomes, were prepared as described above by diluting 3 μg DNA and the desired amount of PEI, each in 50 μL HEPES buffer (20 mM, pH 8.0) prior to mixing. For cross-linking, 2.5 μL of DSP in DMSO (0.1 mg/mL) was added to each sample, lightly vortexed and incubated at RT for 1 h. Various concentrations of diolein/CHEMS liposomes in 50 μL HEPES buffer were then added to each sample, depending on the desired lipid:DNA ratio, and allowed to stand at RT for 15 min. Finally, 50 μL (1 μg DNA/well) of the resulting solution was added to each well of a 24-well plate to evaluate transfection potential.

To analyze luciferase expression, the growth medium was removed from each well and the cells rinsed twice with cold PBS. Next, 200 μL ice cold lysis buffer (0.5% Triton X-100, 100 mM Tris-HCl, 2 mM EDTA, pH 7.8) was added to each well, followed by incubation on ice for 30 min. The lysate was then removed and centrifuged...
for 1 min at 13,000 RPM. Finally, 10 μL of cellular lysate was mixed with 50 μL luciferase substrate (Promega), and the luminescence measured for 10 s on a Mini-Lum luminometer (Bioscan, Inc., Washington, DC). Relative light units were standardized for protein concentration as determined by the BCA protein determination assay using bovine serum albumin standards. Luciferase activity is also expressed as fmol luciferase/mg protein, determined from a standard curve of luciferase activity using recombinant luciferase.

4.3.8 Negative stain transmission electron microscopy (TEM)

DSP cross-linked polyplexes (10:1) and LPDII particles were prepared as described above at an N/P ratio of 6 and a lipid:DNA ratio of 5 (w/w), respectively, with each sample containing 10 μg of plasmid DNA. An aqueous solution of bacitracin was prepared at a concentration of 200 mg/mL, and diluted to a working strength of 10 mg/mL. An aqueous 1 % uranyl acetate solution was also prepared, and was filtered and centrifuged to remove any particulate matter before use. Samples were prepared by placing a 200 mesh size formvar coated copper grid on a drop of bacitracin solution (10 mg/mL) for 1 min, before wicking away excess solution from the grid with filter paper. Next, each grid was placed in contact with the appropriate sample for 3 min, and the excess liquid again wicked away with filter paper. The grid was then subjected to two brief sequential rinses by placing the grid on a drop of water and wicking away excess fluid. Finally, the grid was incubated in 1 % uranyl acetate solution for 2.5 min, excess liquid was removed and the grid was allowed to dry at RT for 5 min. Electron
micrographs were obtained at 60 kV at 100,000x magnification for polyplexes and
diolein/CHEMS liposomes or 45,000x magnification for LPDII particles.
4.4 Results

4.4.1 Effect of reducible cross-linking on the transfection activities of PEI/DNA polyplexes

To evaluate the effect of cross-linking on polyplex-mediated transfection of KB cells in vitro, 25 kDa PEI/pLuc DNA polyplexes were prepared at an N/P (PEI nitrogen:DNA phosphate) ratio of 10, followed by cross-linking with DSP or DTBP at cross-linker:PEI molar ratios ranging from 0 to 100:1. An N/P ratio of 10 was chosen based on previous studies showing efficient gene expression at this ratio (16). Figure 4.2 shows that as the cross-linking ratio with DSP or DTBP increases from 0 (control) to 100:1, luciferase gene expression decreases by approximately 3-4 orders of magnitude. The data further show that transfection is relatively independent of the reagent used for cross-linking, with nearly identical luciferase activity at a cross-linking ratio of 10. However, at higher cross-linking ratios (≥ 20), slightly better gene expression was observed with polyplexes cross-linked with DTBP.

4.4.2 Stability of PEI/DNA polyplexes in the presence of a competing polyanion

Our next goal was to evaluate the stability of polyplexes, either with or without covalent stabilization by cross-linking, in the presence of a competing polyanion. Our initial stability analysis was performed on conventional PEI/DNA polyplexes prepared at a variety of N/P ratios, namely 0 (DNA only), 2, 3, 5 and 10. These polyplexes were exposed to various concentrations of PMAA, an organic polyanion, ranging from 0-100 μM, followed by addition of EtBr and quantitative fluorescence measurements. Detection of EtBr fluorescence is an indication of DNA being accessible for EtBr binding due to

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polyplex dissociation, or at least local decondensation of DNA. The EtBr fluorescence observed upon incubation with free plasmid DNA, in the absence of PEI and PMAA, was used as a reference control (i.e. - 100 % relative fluorescence). As seen in Figure 4.3, the addition of PMAA to free plasmid DNA, at concentrations up to 100 μM, had virtually no effect on fluorescence (N/P = 0). The results also show that in the absence of PMAA, EtBr fluorescence for all polyplexes decreases to ~ 10 % of control, with the exception of polyplexes prepared at an N/P ratio of 2. Polyplexes prepared at this ratio are known to cause incomplete condensation of DNA due to inadequate charge neutralization, which led to an EtBr fluorescence reading of ~ 30 %. As the concentration of PMAA was increased, progressively higher fluorescence readings were observed for all polyplexes, with values eventually approaching 100 % of control at a PMAA concentration of 100 μM. Furthermore, polyplexes prepared at higher N/P ratios appeared more resistant to polyanion disruption, which can be seen in Figure 4.3 from the lower fluorescence readings obtained at high N/P ratios and low PMAA concentrations.

4.4.3 Effect of covalent cross-linking on polyplex stability

Since polyplex stability is apparently compromised by the presence of a competing polyanion, experiments were then performed to evaluate the consequence of cross-linking PEI/DNA polyplexes with DSP, at various DSP:PEI molar ratios, before exposure to PMAA. Polyplexes were prepared at an N/P ratio of 6, and were then cross-linked with DSP at molar ratios of 10, 20, 50 and 100:1. PMAA was included in all samples at a concentration of 100 μM, since that concentration restored nearly 100 % EtBr fluorescence when incubated with uncross-linked polyplexes. As seen in Figure 4.4, the
results show that as the DSP:PEI cross-linking ratio is increased, PEI/DNA polyplexes become increasingly resistant to polyanion challenge, exhibited by the decrease in EtBr fluorescence. In fact, fluorescence readings for polyplexes cross-linked at a ratio of 100:1 decreased by more than 50%. However, when 20 mM dithioerythritol (DTE) was introduced to promote reduction of the intramolecular disulfide bond present in DSP, effectively reversing stabilization, EtBr fluorescence was restored to ~ 90% of control for all samples.

4.4.4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to obtain visual evidence to supplement the quantitative fluorescence readings in Figure 4.4. The underlying principle was the same as for fluorometric analysis (i.e.-EtBr fluorescence is restored upon polyplex dissociation or local decondensation of DNA). However, visualization by electrophoresis provides a basis to discern between dissociation or local DNA decondensation. As seen in lane 4 of Figure 4.5, incubation of uncross-linked polyplexes (N/P = 6) with 100 µM PMAA results in polyplex dissociation, as evidenced by the DNA migration through the gel. The results also show (lanes 5-8) that despite DSP cross-linking, some EtBr fluorescence can be seen in the loading wells. Lanes 9-12 illustrate the effect of DTE reduction of disulfide bonds on DNA migration and EtBr fluorescence. At cross-linking ratios of 10, 20 and 50:1 (lanes 9-11), pLuc DNA regained its fluorescence and was free to migrate through the gel. However, at 100:1 (lane 12), fluorescence was mainly visible in the loading well, with a faint band appearing where plasmid DNA would be expected, despite DTE reduction.

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4.4.5 Transfection of KB cells with various LPDII formulations

Transfection studies were performed to compare the transfection performance between LPDII vectors either with or without covalent cross-linking of polyplexes in KB cells in vitro. Polyplexes were prepared at an N/P ratio of 4, yielding condensed particles with a slight positive charge. Where DSP cross-linking was employed, polyplexes were cross-linked at a molar ratio of 10:1 (DSP:PEI). Figure 4.6 displays the transfection results from a series of four different LPDII formulations, along with results from a 1 mM free folate competition study for the folate receptor-targeted formulation. Specifically, the formulations tested included conventional diolein/CHEMS LPDII particles without DSP cross-linking, LPDII including covalently cross-linked polyplexes, LPDII prepared with PEG containing liposomes, and receptor-targeted LPDII particles containing a lipid anchored folate moiety. As indicated, all polyplexes were PEI/DNA complexes, and the anionic liposome component was composed of diolein/CHEMS prepared at a molar ratio of 6:4. Inclusion of PEG-DSPE, for steric stabilization, or folate-PEG-Chol, for receptor targeting, was achieved by adding 0.5 mol % of the appropriate lipid anchored molecule during preparation of the diolein/CHEMS liposomes.

Figures 4.6A and 4.6B depict the luciferase gene expression achieved with each LPDII formulation as a function of lipid:DNA (w/w) ratio. Transfection levels mediated by all formulations increased initially, before achieving peak luciferase activities at a lipid:DNA ratio of 2.5. All formulations then exhibited a subsequent decrease in luciferase activity at a lipid:DNA ratio of 5. At ratios beyond 5, luciferase activities appeared to level off, with the exception of LPDII prepared without DSP cross-linking, which displayed steadily decreasing luciferase activities with increasing lipid:DNA ratio.
From a comparison of conventional diolein/CHEMS LPDII vectors and LPDII that include DSP cross-linked polyplexes, it can be seen that cross-linking at a ratio of 10:1 led to an ~ 2-16-fold decrease in gene expression at lipid:DNA ratios ≤ 7.5, but that similar luciferase activities were achieved at ratios beyond 10. Inclusion of PEG-DSPE at 0.5 mol % appeared to consistently hinder transfection levels, reducing luciferase activities ~ 2-5-fold, compared to LPDII containing cross-linked polyplexes prepared without PEG-DSPE. In contrast, LPDII containing folate-PEG-Chol exhibited higher gene expression at all lipid:DNA ratios tested, compared with LPDII vectors containing PEG-DSPE. In fact, luciferase activities for LPDII containing folate-PEG-Chol were ~ 5-10 times greater than the PEG-DSPE formulation at lipid:DNA ratios between 1-5, with decreasing differences at higher ratios. Compared with cross-linked LPDII without PEG-DSPE, inclusion of 0.5 mol % folate-PEG-Chol increased luciferase activities 3-4-fold between lipid:DNA ratios of 1-5, and, again, led to decreasing differences at high lipid:DNA ratios. Inclusion of 1 mM free folate in the growth media during transfection with LPDII vectors containing folate-PEG-Chol led to luciferase activities nearly identical to those achieved in the absence of free folate, with the exception of an ~ 4.5-fold increase in activity at a lipid:DNA ratio of 2.5.

4.4.6 Effect of free folate on the transfection activity of LPDII formulations

In contrast to the observed results, luciferase activities were expected to decrease for LPDII containing folate-PEG-Chol in the presence of 1 mM free folate, due to competition for the folate receptor. However, it is known that addition of free folate to the growth media during transfection increases transfection levels mediated by PEI/DNA
polyplexes (17). Therefore, transfection studies to compare the effect of free folate on gene expression were repeated with conventional diolein/CHEMS LPDII vectors, LPDII formulated with PEG-DSPE and LPDII containing folate-PEG-Chol. All formulations contained DSP cross-linked polyplexes at a ratio of 10:1 (DSP:PEI). Free folate was included in the growth media at a concentration of 1 mM, as in earlier transfection studies, with additional concentrations of 2 mM and 5 mM included for the folate receptor-targeted formulation. Figure 4.7 shows that inclusion of 1 mM free folate in the growth media increased luciferase activities 3-4-fold for diolein/CHEMS LPDII and LPDII containing folate-PEG-Chol, but had little effect on LPDII containing PEG-DSPE. Furthermore, increasing the free folate concentration to 2 mM during transfection with folate receptor-targeted LPDII vectors decreased luciferase activity, in comparison to 1 mM free folate, and achieved similar transfection levels to those observed in the absence of free folate. Increasing the free folate concentration to 5 mM led to a further decrease in luciferase activity, which was ~ 6-fold lower than transfection levels obtained without free folate present in the growth media.

4.4.7 Negative stain transmission electron microscopy

Finally, negative stain transmission electron microscopy was performed to obtain evidence confirming the association between diolein/CHEMS liposomes and cross-linked polyplexes (Figure 8). Panel A shows PEI/DNA polyplexes cross-linked with DSP at a molar ratio of 10:1. Polyplexes appeared as small dark spherical particles ranging in diameter from ~ 70-100 nm. Panel B shows diolein/CHEMS liposomes prepared at a molar ratio of 6:4, which appeared as white spherical particles having a diameter between
100-250 nm. Finally, panels C and D depict LPDII particles formed at a lipid:DNA ratio of 5. These complexes were manifested as aggregated structures, roughly spherical in shape, with diameters ranging between 300-800 nm.
4.5 Discussion

Currently, the primary limitation in the development of clinically useful non-viral gene therapy protocols is the lack of safe and efficient non-viral vectors for \textit{in vivo} gene delivery. Here, we evaluate a novel approach to improve the stability and transfection potential of polyplex and LPDII mediated gene transfer. We showed that cross-linking PEI/DNA polyplexes stabilizes these complexes against polyanion disruption, and that cross-linking is reversible upon reduction by DTE. We further demonstrated that LPDII transfection vectors mediate variable levels of transfection, depending on the presence of DSP cross-linking and the incorporation of PEG-DSP or folate-PEG-Chol as a component of the diolein/CHEMS liposomes used for LPDII formation. It was also observed that the presence of 1 mM free folate in the transfection media has a surprisingly beneficial effect on transfection, but that this effect was dose dependent for LPDII vectors containing folate-PEG-Chol. Finally, we provide evidence, by way of electron micrographs, that diolein/CHEMS liposomes and DSP cross-linked polyplexes form aggregated complexes under our experimental conditions.

Initial transfection studies with cross-linked polyplexes were performed to assess the effects of DSP or DTBP cross-linking of PEI/DNA polyplexes on luciferase reporter gene expression. Branched, 25 kDa PEI was used as the condensing agent because previous studies indicate that PEI is an effective transfection agent both \textit{in vitro} (16, 18) and \textit{in vivo} (19-21). Transfection results indicated that cross-linking polyplexes with DSP or DTBP, after DNA condensation, leads to a reduction in gene expression in KB cells, with significantly lower luciferase activities (3-4 orders of magnitude) observed at higher cross-linking ratios. These results were not surprising, since increasing the cross-linking
ratio would be expected to increase polyplex stability. While increased stability may be beneficial prior to cellular uptake, ultimately the DNA has to be accessible by the nuclear transcription machinery, thus mandating polyplex dissociation and DNA decondensation preceding gene expression. High cross-linking ratios would require more extensive intracellular reduction of disulfide bonds present in the cross-linking reagents in order to reverse stabilization, which may ultimately limit gene expression.

Comparing the two cross-linking reagents, we found transfection activity to be slightly higher with DTBP cross-linked polyplexes, at cross-linking ratios ≥ 20. However, in our experience, DTBP is less reactive toward PEI, compared with DSP, under the chosen reaction conditions. The slightly greater transfection levels mediated by DTBP cross-linked polyplexes at higher cross-linking ratios may be a reflection of less extensive cross-linking, and consequently a lower reduction in gene expression. Alternatively, the retention of positive charge after DTBP cross-linking may promote more efficient gene transfer. In the end, DSP was used to cross-link polyplexes for inclusion in LPDII vectors because cross-linking with DSP can be accomplished with a shorter reaction time, and it is possible to monitor the appearance of NHS from DSP spectrophotometrically to gauge reaction progress. In addition, there were relatively small differences in transfection levels between DSP and DTBP cross-linked polyplexes, especially at a cross-linking ratio of 10:1.

While it was important to understand the effect of cross-linking on transfection levels, it was equally important to investigate the stability of cross-linked polyplexes, in order to justify covalent stabilization. Therefore, polyplex stability was evaluated in the presence of a competing polyanion, namely PMAA, which should mimic the effect of
polyanionic liposomes on polyplex integrity during LPDII formation. Anionic liposomes themselves were not used as the competing polyanion because lipid concentrations utilized for LPDII formulation interfered with EtBr fluorescence detection. Initially, we were interested in determining whether uncross-linked polyplexes were susceptible to dissociation from polyanion disruption as a function of N/P ratio. Therefore, polyplexes were prepared at several N/P ratios, and incubated with various PMAA concentrations, followed by EtBr fluorescence analysis. This analysis is based on the concept that a large increase in fluorescence is observed upon intercalation of DNA by the phenanthridium moiety of EtBr (22). Condensed DNA, like the DNA present in polyplexes prepared with a net positive charge, will exclude EtBr from intercalating between base pairs, thus providing minimal fluorescence. In contrast, uncondensed DNA, resulting from local decondensation of DNA or polyplex dissociation, will permit EtBr intercalation to yield a sharp increase in EtBr fluorescence. The increase in fluorescence will be proportional to the amount of DNA available for intercalation. Our results showed that polyplexes exposed to PMAA exhibit an increase in EtBr fluorescence, and that fluorescence was directly related to PMAA concentration and inversely related to the N/P ratio. This result suggests that conventional polyplexes are susceptible to polyanion disruption, and that polyplexes prepared with large N/P ratios are more stable than those prepared with low ratios.

We anticipated that reversible cross-linking would stabilize polyplexes, and that reduction of the intramolecular disulfide bond present in DSP would restore polyplex susceptibility to polyanion disruption. Therefore, we cross-linked PEI/DNA polyplexes at various DSP:PEI molar ratios and evaluated EtBr fluorescence in the presence of 100
μM PMAA, either with or without 20 mM DTE. The results showed that increasing the
cross-linking ratio led to a decrease in EtBr fluorescence, suggesting more stable
polyplexes at higher cross-linking ratios. Reduction with 20 mM DTE was found to
restore fluorescence levels to ~90% of control. These results are useful in
demonstrating reversible covalent stabilization with DSP. Furthermore, we believe it is
reasonable to infer that polyanionic liposomes would have a similar effect on polyplex
integrity, yet recognize that polymers such as PMAA may interact somewhat differently
with polyplexes. Therefore, it should be noted that these results do not provide actual
evidence regarding the interaction between polyplexes and diolein/CHEMS liposomes.
Nevertheless, although these results were considered positive in terms of demonstrating
stabilization and cross-linking reversibility, they also introduced a dilemma in choosing
the appropriate cross-linking ratio of polyplexes for incorporation into LPDII transfection
particles. Earlier transfection results with cross-linked polyplexes showed a minimal
reduction in gene expression if the cross-linking ratio was relatively low. However, the
stability results showed that EtBr fluorescence was still ~75-80% of control for
complexes cross-linked at the same molar ratio. Therefore, the degree by which DSP
might have contributed to cross-linking stability was unclear.

Consequently, polyanion disruption of cross-linked polyplexes was repeated as
described above, but the samples were instead visualized by agarose gel electrophoresis.
This assay provided a basis to distinguish between polyplex dissociation and local
decondensation of DNA. With polyplex dissociation, free plasmid DNA would be
expected to migrate through the gel at a rate proportional to its size, but with local
decondensation of DNA, fluorescence would likely be observed in the loading well. This
stems from the fact that DNA migration through the agarose gel is based on electrostatic attraction between polyanionic DNA and a positively charged anode. However, when DNA is present in the form of a polyplex, the negative charge is neutralized by the cationic polymer, consequently precluding its migration through the gel. In addition, the size increase resulting from the presence of the high molecular weight PEI in the polyplex would further hinder DNA migration. Ultimately, the electrophoresis results suggested that low cross-linking ratios are adequate to prevent dissociation, but will not completely prevent local DNA decondensation. This was shown in Figure 4.5 by DNA migration with uncross-linked polyplexes in the presence of 100 µM PMAA (lane 4), but after cross-linking with DSP, fluorescence was only visible in the loading wells (lanes 5-9). These results provided a logical basis for cross-linking polyplexes at a DSP:PEI molar ratio of 10:1 for incorporation into LPDII vectors. Specifically, this ratio was chosen because preliminary transfection results using polyplexes cross-linked at 10:1 showed a minimal reduction in gene expression, compared with uncross-linked polyplexes, and agarose gel analysis further showed that these complexes remained associated even with relatively strong polyanion challenge (100 µM PMAA).

To delineate the effects on gene expression, the transfection potential of four related LPDII vectors was evaluated in KB cells in vitro. PEI/DNA polyplexes were formed at an N/P ratio of 4, and, where indicated, cross-linked with DSP at a DSP:PEI molar ratio of 10:1. An N/P ratio of 4 was utilized because this ratio mediates complete condensation of plasmid DNA to form positively charged complexes, yet minimizes free PEI in solution not participating in polyplex formation (19, 23). If free PEI were present in the solution after polyplex formation, DSP could react preferentially with free PEI and
reduce polyplex stabilization by consuming DSP. The anionic liposomes used for LPDII formation were composed of a novel serum-resistant formulation consisting of diolein/CHEMS (6:4 mol/mol). The four LPDII formulations tested for luciferase reporter gene expression included conventional LPDII vectors without DSP cross-linking, LPDII including covalently cross-linked polyplexes, LPDII prepared with PEG containing liposomes, and receptor-targeted LPDII vectors containing a lipid anchored folate moiety.

From the transfection results shown in Figure 4.6A, the first comparison can be made between LPDII vectors containing DSP cross-linked polyplexes and those without cross-linking. The transfection results show that LPDII vectors with covalent cross-linking mediated lower luciferase activities at lipid:DNA ratios \( \leq 7.5 \), yet similar transfection levels at lipid:DNA ratios \( \geq 10 \). Results from the transfection studies using cross-linked polyplexes for gene transfer, illustrated in Figure 4.2, showed that DSP cross-linking inhibited gene expression, and this effect may contribute to the observed decrease in transfection activity mediated by LPDII vectors containing cross-linked polyplexes. Alternatively, LPDII particle formation may be deleteriously altered by the presence of DSP, or more likely by the elimination of positive charges on PEI after reaction with DSP, since LPDII particles largely depend on electrostatic attraction for complex formation. LPDII vectors prepared in the absence of cross-linking achieve a peak luciferase activity at a lipid:DNA ratio of 2.5, and then display steadily decreasing transfection levels. In contrast, LPDII containing cross-linked polyplexes display a more level luciferase activity profile over the entire range of lipid:DNA ratios. This observation suggests that particle assembly may be saturable at low lipid:DNA ratios,
again possibly due to the elimination of positive charge after DSP cross-linking. Despite the disparity in transfection activity between these two formulations, LPDII particles containing cross-linked polyplexes did mediate significant levels of transfection, and, therefore, may prove useful under *in vivo* conditions where complex stability could be a more dominant factor.

Inclusion of PEG-DSPE at 0.5 mol % into the LPDII formulation containing DSP cross-linking resulted in a further reduction in gene expression at all lipid:DNA ratios tested, compared with the cross-linked formulation in the absence of PEG-DSPE. This effect may be the result of reduced interaction between polyplexes and liposomes, or a reduction in cellular uptake. Since particle size is an important determinant for *in vitro* transfection, with smaller particles achieving lower transfection, it could also be postulated that incorporation of PEG-DSPE reduces any interactions between transfection complexes in solution that might cause an increase in particle size, thus reducing gene expression.

The contribution of folate receptor targeting, through incorporation of 0.5 mol % folate-PEG-Chol, can be seen in Figure 4.6B by comparing the transfection activity of folate receptor-targeted LPDII vectors with LPDII formulated with PEG-DSPE. Based on that comparison, the data suggest that attachment of folate to the distal end of a PEG linker does mediate an increase in luciferase activity at all lipid:DNA ratios, but especially at ratios ≤ 5. However, to confirm that folate receptor-mediated endocytosis contributes to increased gene expression, a folate receptor competition study was performed by introducing 1 mM free folate in the growth media during transfection. The results, also shown in Figure 4.6B, were inconsistent with our expectations that luciferase
activity would decrease in the presence of competition by free folate. However, as indicated earlier, previous studies in our lab have shown that inclusion of free folate in the growth media during transfection increases the transfection activity of PEI-based polyplexes. In effect, transfection studies were performed to determine if free folate had a similar effect on LPDII particles prepared at a lipid:DNA ratio of 2.5. We found that inclusion of 1 mM free folate in the growth media during transfection increased the transfection activity of LPDII containing cross-linked polyplexes and LPDII containing folate-PEG-Chol, but had little effect on the transfection activity of LPDII formulated with PEG-DSPE. To determine if higher concentrations of free folate could inhibit the transfection activity of folate receptor-targeted LPDII vectors, similar transfection studies were performed in the presence of 2 mM and 5 mM free folate. The results showed that luciferase activity was inversely related to the concentration of free folate in the growth media. The mechanism responsible for the increased gene expression in the presence of 1 mM free folate has not been determined, and it is curious that LPDII containing PEG-DSPE were relatively unaffected. Regardless, we conclude that inclusion of folate-PEG-Chol in LPDII vectors mediates greater luciferase activities compared with LPDII formulated with PEG-DSPE. Furthermore, the presence of 1 mM free folate in the growth media during transfection apparently has a beneficial effect on transfection activity, and, therefore, does not offer conclusive evidence that increased gene expression from inclusion of folate-PEG-Chol is truly receptor mediated. The fact that higher concentrations of folate do reduce observed transfection levels is consistent with expectations, but 5 mM free folate is orders of magnitude greater than the $K_d$ for folic acid, $10^{-9}$-$10^{-10}$ M. Although the concentrations cited below are ~10-times lower, similar
observations have been reported previously that show the IC\textsubscript{50} for folate receptor-targeted liposomes is 0.2-0.4 mM (10, 15). The high binding strength of these liposomes has been attributed, in part, to the high binding affinity achieved by multivalent attachment of multiple targeting ligands.

Finally, negative stain transmission electron micrographs of DSP cross-linked polyplexes, diolein/CHEMS liposomes and LPDII vectors containing covalently cross-linked polyplexes were obtained to gather size and structural information, and to provide evidence of LPDII complex assembly. The images shown in Figure 4.8A display cross-linked polyplexes that appear as dark spherical particles having a diameter between 70-100 nm. This micrograph is consistent with previously published observations of PEI-based polyplexes (24, 25). Figure 4.8B displays images of diolein/CHEMS liposomes, which appear as white spherical particles smaller than 250 nm. To our knowledge, these are the first published images of diolein/CHEMS liposomes. Figures 4.8C and 4.8D portray LPDII particles composed of diolein/CHEMS liposomes and cross-linked polyplexes. The diameter of these particles was somewhat variable, but was generally larger than either the polyplexes or liposomes. The LPDII particles displayed in Figure 4.8 had diameters between 500-700 nm. This size information suggests that the increased transfection performance of LPDII particles may in part be a result of increased particle size, which would promote sedimentation of transfection particles \textit{in vitro}. In addition, these images appear consistent with the hypothesis that LPDII complexes are composed of aggregated complexes of polyplex and anionic liposomes. However, since the polyplex would theoretically be located inside this complex, and the LPDII particles were not visualized in cross-section, it cannot be ruled out that the particles observed in
Figures 4.8C and 4.8D are simply aggregates of liposomes. The fact that these types of particles were not observed when viewing diolein/CHEMS liposomes, however, does provide some support to the notion that the complexes observed in Figure 4.8 are LPDII particles.

To our knowledge, this is the first report describing covalent stabilization of PEI/DNA polyplexes for subsequent inclusion in LPDII vectors. The concept of reversible cross-linking via reducible disulfide bonds has been investigated by other groups, but only in the context of polyplex mediated gene transfer (26, 27). The underlying theory behind this approach is that reversible disulfide cross-linking will confer stability to transfection complexes until intracellular reduction of the disulfide bonds takes place. If the transfection vectors are in fact able to gain access to the reducing environment of the cytoplasm, it is plausible that reduction of these bonds will occur, possibly through interaction with reduced glutathione. Accordingly, the data presented here indicate that the effects of cross-linking are reversible upon disulfide bond reduction with DTE in vitro. Furthermore, our results show that polyplexes are more stable in terms of dissociation in the presence of a competing polyanion after DSP cross-linking, and that inclusion of cross-linked polyplexes into LPDII vectors mediates variable transfection, depending on the lipid:DNA ratio and the presence of PEG-DSPE or folate-PEG-Chol. The combination of serum resistance conferred by the diolein/CHEMS liposomes and the increased stability owed to cross-linking may make LPDII vectors incorporating these features more stable in the systemic circulation after intravenous delivery. This improved stability may ultimately translate into higher gene expression and improved therapeutic effects. Based on these results, further evaluation of
the transfection properties of covalently cross-linked LPDII vectors in an animal model is warranted.
Figure 4.1 Proposed reaction scheme for covalent cross-linking of PEI/DNA polyplexes with cross-linking reagents DSP and DTBP. DSP cross-linking is illustrated above the schematic polyplex, while DTBP cross-linking is shown below. Also shown is the effect of disulfide bond reduction from 20 mM dithioerythritol (DTE).
Figure 4.2 Effect of reducible cross-linking on the transfection activities of PEI/DNA polyplexes. Polyplexes were prepared at an N/P ratio of 10. Polyplexes were then cross-linked with DSP or DTBP at cross-linker:PEI molar ratios of 0, 10, 20, 50 and 100:1. The transfection activity of the covalently cross-linked polyplexes was then evaluated in KB cells in vitro. Cells were exposed to transfection vectors for 4 h in serum-free media, and luciferase activity was assayed 24 h later.
Figure 4.3 EtBr fluorescence from interactions with PEI/DNA polyplexes in the presence of a competing polyanion (PMAA). Polyplexes were prepared at N/P ratios of 0, 2, 3, 5 and 10, and exposed to various concentrations of PMAA up to 100 μM. EtBr was added to each sample at a concentration of 0.4 μg/mL, and fluorescence was measured at excitation and emission wavelengths of 523 nm and 602 nm, respectively.
Figure 4.4 EtBr fluorescence from interactions with DSP cross-linked PEI/DNA polyplexes in the presence of 100 μM PMAA, with or without disulfide bond reduction with DTE. Polyplexes were prepared at a N/P ratio of 6, and cross-linked with DSP at DSP:PEI molar ratios of 0, 10, 20, 50 and 100:1. Cross-linked polyplexes were then exposed to 100 μM PMAA for 30 min, with or without 20 mM DTE. EtBr was added to each sample at a concentration of 0.4 μg/mL, and fluorescence was measured at excitation and emission wavelengths of 523 nm and 602 nm, respectively.
Figure 4.5 Stability of covalently cross-linked polyplexes analyzed by agarose gel electrophoresis. Polyplexes were prepared at a N/P ratio of 6, and cross-linked with DSP at DSP:PEI molar ratios of 10, 20, 50 and 100:1. Cross-linked polyplexes were then exposed to 100 μM PMAA for 30 min, with or without 20 mM DTE, and analyzed by agarose gel electrophoresis (0.5 μg DNA/lane). Lane 1 is Hind III digested phage λ DNA, used as a size marker; lane 2 is pLuc DNA only; lane 3 is PEI/pLuc polyplex (N/P = 6); lane 4 is PEI/pLuc polyplex with 100 μM PMAA; lanes 5-8 are PEI/pLuc polyplexes cross-linked with DSP at 10:1, 20:1, 50:1 and 100:1, respectively, with 100 μM PMAA; lanes 9-12 are identical to lanes 5-8, except for the addition of 20 mM DTE.
Figure 4.6 Transfection of KB cells with various LPDII formulations as a function of lipid:DNA ratio. The lipid:DNA ratios tested were 0 (cross-linked or uncross-linked polyplex alone), 2.5, 5, 7.5, 10 and 15. All formulations contain PEI/pLuc DNA polyplexes prepared at a N/P ratio of 4. Except where noted in the figure, all polyplexes were cross-linked with DSP at a DSP:PEI molar ratio of 10:1. (A) Diolein/CHEMS liposomes were prepared at a molar ratio of 6:4, and where indicated (B) 0.5 mol % PEG-DSPE or folate-PEG-Chol was included.

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Figure 4.7 Effect of free folate on the transfection activity of the LPDII formulations containing DSP cross-linked polyplexes. Free folate (FF) was included at 1, 2 or 5 mM in the growth media during transfection. All polyplexes used in LPDII preparation were cross-linked with DSP at a DSP:PEI molar ratio of 10:1. The three LPDII formulations tested include diolein/CHEMS (D/C), diolein/CHEMS/PEG-DSPE (D/C/PEG or PEG) and diolein/CHEMS/folate-PEG-Chol (D/C/Fol or Fol). Diolein/CHEMS liposomes were prepared at a molar ratio of 6:4, and where indicated 0.5 mol % PEG-DSPE or folate-PEG-Chol was included.

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Figure 4.8 Negative stain transmission electron microscopy. DSP cross-linked polyplexes (10:1) and LPDII particles were prepared at an N/P ratio of 6 and a lipid:DNA ratio of 5 (w/w), respectively, with each sample containing 10 μg of plasmid DNA. Electron micrographs were obtained at 60 kV. (Panel A) DSP cross-linked PEI/DNA polyplexes at 100,000x magnification, (Panel B) Diolein/CHEMS liposomes (6:4, mol/mol) at 100,000x magnification, (Panels C & D) DSP cross-linked LPDII vectors at 45,000x magnification. In panels A & B, the bar = 100 nm; in panels C & D, the bar = 200 nm.
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CHAPTER 5

SUMMARY AND FUTURE PERSPECTIVES

This dissertation describes the development and application of strategies to improve the stability and transfection performance of non-viral vectors for gene delivery. The overall goal was to design transfection vectors that mediate greater levels of gene expression, since this is currently the primary limiting factor in non-viral gene therapy. Vectors were designed to incorporate features that are theoretically favorable for intravenous administration, and to alleviate some of the deficiencies of current non-viral vectors. The formulations described in this dissertation are modifications of polyethylenimine (PEI)-based polycation/DNA complexes (polyplexes) and liposome-entrapped polycation-condensed DNA (LPD).

Chapter 2 described the development of polyplexes comprised of low molecular weight PEI cross-linked with either dithiobis[succinimidylpropionate] (DSP) or dimethyl 3,3′-dithiobispropionimidate 2 HCl (DTBP). The goal was to take advantage of the high transfection activity of high molecular weight PEI, by utilizing covalently cross-linked conjugates of low molecular weight PEI for DNA condensation and transfection, and the reduced toxicity of low molecular weight PEI. The reduction in toxicity would theoretically arise from the reduction of the disulfide bonds present in DSP and DTBP.
thereby, promoting dissociation of the cross-linked PEI conjugates to yield low molecular weight PEI. Furthermore, it was reasoned that the reversion of high molecular weight PEI to low molecular weight PEI might enhance gene expression by reducing the association between PEI and DNA, thereby, allowing easier access to the DNA by molecules required for transcription.

The results described in chapter 2 demonstrated that polyplexes formulated with cross-linked PEI can mediate transfection levels similar to those observed using high molecular weight PEI. However, transfection was dependent on the cross-linking reagent, the PEI nitrogen/DNA phosphate (N/P) ratio, and the molar cross-linking ratio utilized. It was also shown that transfection using cross-linked polyplexes led to a decrease in toxicity, as evidenced by greater protein levels from samples transfected with cross-linked polyplexes, compared with high molecular weight PEI. Finally, analysis by agarose gel electrophoresis showed that in the presence of a reducing agent the disulfide bonds present in DSP and DTBP could be cleaved. It was also shown that reduction of the disulfide bonds promoted dissociation of PEI and DNA, as seen by the migration of plasmid DNA through an agarose gel.

Covalent cross-linking, employed as a strategy to modify non-viral vectors, has shown some potential to improve the transfection properties of polyplex mediated gene delivery. The future development of this approach would likely be improved by designing methods to characterize the extent of cross-linking, in terms of the polydispersity of the resulting polymer molecular weights and the effects on the physical properties of the cross-linked polymers, including surface charge and buffering capacity. This could then be extended to define how altering these properties will affect the
interactions between PEI and plasmid DNA. Furthermore, although transfection using cross-linked PEI did not lead to an increase in gene expression, compared with 25 kDa PEI, cross-linked polyplexes did appear to decrease toxicity. It would be interesting to determine if measuring total cellular protein after transfection correlates well with more rigorous methods for determining cellular toxicity. The mechanism of toxicity could then be characterized to determine if cell death could be minimized by altering the gene delivery formulation.

Chapter 3 explains the development of a novel diolein-based anionic pH-sensitive liposome formulation for incorporation into LPDII transfection vectors. These studies were performed to determine if altering the lipid composition of the anionic liposomes used for LPDII preparation would avoid the significant reduction in transfection activity observed using DOPE-based liposomes in the presence of serum. The rationale was that vectors being developed for administration by the intravenous route would ultimately need to retain their transfection properties in serum, since these vectors would obviously be exposed to serum upon their introduction into the blood circulation. It was also important that the new diolein-based liposomes display pH-sensitivity in order to facilitate endosomal escape into the cytoplasm after internalization into endosomal compartments. Finally, LPDII vectors formulated with diolein-based liposomes had to mediate transfection levels that were comparable or superior to previous LPDII formulations and other common transfection agents.

The results presented in chapter 3 showed that liposomes formulated with diolein and cholesteryl hemisuccinate (CHEMS) at a molar ratio of 6:4 displayed adequate stability at neutral pH and destabilization and aggregation at acidic pH. This was shown using an
assay that measures calcein fluorescence after release from liposomes and by particle size analysis. The results also showed that LPDII vectors prepared with PEI/DNA polyplexes and diolein/CHEMS liposomes mediated relatively high levels of gene expression. Gene expression was dependent on both the N/P ratio of the PEI polyplexes and the lipid to DNA (L/D) ratio of LPDII vectors. It was further shown that diolein/CHEMS liposomes facilitate endosomal release by replacing PEI with poly-L-lysine (PLL) as the DNA condensing agent. Unlike PEI, PLL is unable to promote endosomal release of transfection complexes into the cytoplasm. Since the DNA contained in the transfection complexes must gain access to the cytoplasm and eventually the nucleus for transcription, the observed endosomal escape and subsequent gene expression was attributed to the action of the diolein/CHEMS liposomes. Transfection studies in media containing up to 50 % serum demonstrated that LPDII vectors formulated with diolein/CHEMS liposomes not only retain their transfection activity, but appear to mediate more efficient gene delivery in the presence of serum. This was an important observation in terms of validating the use of diolein as component of LPDII vectors. Finally, it was shown that diolein-based LPDII vectors mediate transfection levels that were superior to other selected common transfection vectors.

Due to the fact that LPDII vectors are formed by random electrostatic interactions between cationic polyplexes and anionic liposomes, characterization of this association in terms of the saturability of particle formation, stability of the resultant particles and conformational changes induced in either the polyplex or liposomes after association may offer some insight as to how these vectors could be improved. It would also be useful to determine how serum components interact with LPDII particles. These interactions
might be critical in understanding the transfection properties of these vectors in an \textit{in vivo} model. In addition, defining how LPDII particles interact with cells at the cell surface, the mechanism of internalization, and the subsequent intracellular distribution could yield valuable information regarding the factors limiting LPDII-mediated gene expression.

The LPDII concept for gene delivery was extended in chapter 4 to include covalent cross-linking of the polyplexes utilized for LPDII vector assembly, incorporation of lipid anchored PEG molecules into the diolein/CHEMS liposomes, and inclusion of lipid anchored folate targeting-moieties attached to the distal ends of PEG spacers. DSP and DTBP were used to cross-link 25 kDa PEI/DNA polyplexes in an effort to achieve reversible stabilization of these complexes. It was thought that reversible stabilization of polyplexes for incorporation into LPDII vectors would prevent dissociation of the polyplexes in the presence of anionic liposomes. However, when exposed to the reducing environment of the cytoplasm, this stabilization would be reversed, thus, releasing the DNA for transcription. Folate was introduced into the LPDII formulation as a targeting-ligand to promote receptor-mediated endocytosis of transfection vectors. The rationale was that increasing internalization of transfection vectors would lead to higher levels of gene expression. Inclusion of PEG into the diolein/CHEMS liposomes was used as a negative control for the folate receptor-targeted formulation. However, the effect of PEG on transfection was also of interest, since PEG has been shown to increase the circulation half-life of liposomes in the systemic circulation.

The results presented in chapter 4 showed that the presence of polymethacrylic acid, an organic polyanion, compromised the integrity of PEI/DNA polyplexes, which was determined using an ethidium bromide fluorescence assay. Results also showed that
polylexes prepared at higher N/P ratios were more resistant to this effect. These results confirmed the suspicion that polylexes are susceptible to polyanion-mediated disruption. Incorporation of covalent cross-linking was shown to stabilize polylexes against dissociation, but that stabilization was reversible after reduction of the disulfide bonds present in the cross-linking reagents. To discriminate between local decondensation of DNA and dissociation of polylexes, polylexes were analyzed by agarose gel electrophoresis. The results further showed that cross-linking polylexes with DSP or DTBP led to a decrease in reporter gene expression. However, this decrease was more pronounced at high molar cross-linking ratios. Fortunately, low cross-linking ratios did not cause a large reduction in transfection activity, but were capable of stabilizing polylexes against dissociation. Subsequent inclusion of these cross-linked polylexes into LPDII vectors formulated with diolein/CHEMS liposomes led to a decrease in transfection activity, especially at low L/D ratios. In comparison with PEG, inclusion of folate attached to the distal end of a PEG linker into the diolein/CHEMS liposomes led to an increase in gene expression at all L/D ratios tested. However, this result is ambiguous, due to observations that 1 mM free folate increased the transfection activity of some LPDII vectors, including folate receptor-targeted vectors. Higher folate concentrations did inhibit transfection by folate receptor-targeted LPDII vectors, but this did not provide definitive evidence of receptor-mediated enhancement of internalization. Finally, transmission electron micrographs were obtained to gather size and structural information about cross-linked LPDII vectors. The images presented in chapter 4 display LPDII particles as aggregated structures with diameters ranging from approximately 600-800 nanometers.
Despite the reduction in gene expression observed after the incorporation of covalent cross-linking into LPDII vectors, this strategy may still prove useful in the development of non-viral vectors for gene delivery. Studies that determine how cross-linking reduces the transfection efficiency of LPDII vectors would facilitate the development of modified formulations that yield higher gene expression. It might be useful to determine whether cross-linking prevents dissociation of PEI and DNA intracellularly, thereby, inhibiting transcription, or if LPDII vector assembly is compromised by the elimination of positive charges on polyplexes after DSP cross-linking. Elimination of positive charges would theoretically reduce the electrostatic interactions between the polyplexes and anionic liposomes used for LPDII formation. Alternatively, the reduction in transfection efficiency might result from a combination of these factors, in which case the relationship between them should be defined. In addition, studies to elucidate how free folate contributes to LPDII-mediated transfection will also be necessary to determine if incorporation of folate into diolein/CHEMS liposomes does promote receptor-mediated endocytosis. Folate has been shown to increase the transfection activity of PEI polyplex-mediated transfection, but the mechanism regarding this enhancement of gene expression is currently unknown. Finally, if LPDII vectors are to be utilized for intravenous gene delivery, it might be necessary to reduce the size of these vectors or develop a method to isolate smaller LPDII complexes for injection.

Several novel strategies for the improvement of non-viral transfection vectors have been presented in this dissertation. The results show that each of these approaches has potential advantages and disadvantages. Eventually, testing these strategies in an in vivo model will determine which approach, if any, warrants further development and
optimization. If the strategies introduced in this dissertation prove to be incompatible with intravenous administration, they may find use in other gene therapy applications. Some of these applications include pulmonary gene delivery, gene delivery to the nervous system, or intratumoral injection. Due to the relatively high transfection efficiency of diolein-based LPDII vectors, it is conceivable that these vectors could be used for \textit{ex vivo} gene delivery, whereby cells are transfected \textit{in vitro} before being reintroduced into patients. This approach could alleviate concerns of viral contamination in samples after viral gene delivery to cells for reimplantation.

Gene therapy could very well be the most powerful concept ever developed for the treatment of genetic disease. This approach has the potential to cure diseases at their root, instead of merely treating disease symptoms. In order for gene therapy to be successful, scientists must continue to learn more about cell biology, the human genome, the proteins it encodes, and the complex regulatory mechanisms governing gene expression. This knowledge must then be applied to choose better targets for disease treatment, improve nuclear uptake of DNA, extend the duration of gene expression, and increase the levels of gene products. In order to treat complex genetic diseases like cancer, it may also be necessary to strictly regulate the expression of DNA introduced for the purpose of gene therapy. It might also be necessary to introduce multiple genes to effectively treat some diseases, and, furthermore, to control the expression of these genes in relation to one another. Currently, these ideas are nothing but fanciful speculation, but if the components governing the complex biochemical interactions in cells are eventually elucidated, and given time they should be, then it is theoretically possible to control and manipulate that system for the purpose of curing disease. At this point, if gene therapy is
going to succeed as a therapeutic treatment option, it will likely be for a monogenic
disease, such as hemophilia, where only a single dysfunctional gene product contributes
to the disease state. Some progress has been achieved towards this end, and even if gene
therapy never realizes its full potential, it seems probable that it will have some
therapeutic applications for the treatment of genetic diseases in the future.
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