Combinatorial Study of Lipid-like Materials for Gene Delivery

A thesis

submitted by

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In partial fulfillment of the requirements

for the degree of

Master of Science (M.S.)

in

Chemical Engineering

TUFTS UNIVERSITY

2012 May

ADVISER: Qiaobing Xu, PhD

ABSTRACT

The evaluation of a library of lipidoids for *in vitro* DNA delivery through a facile combinatorial approach was investigated. The mild reactions between amines and acrylamides, acrylates, or epoxides enabled the construction of a library to screen for efficient gene delivery carriers.

In Chapter 2, a combinatorial library of lipidoids was constructed and studied for *in vitro* gene delivery. The library of lipidoids was synthesized by reacting commercially available amines with lipophilic acrylates, acrylamides, or epoxides. Lipidoids derived from amine **86** (N,N-Bis(2-hydroxyethyl)ethylene diamine) and amine **87** (N-(3-aminopropyl)diethaneamine) showed high efficiency in DNA delivery, some with a higher transfection efficiency than Lipofectamine 2000, a commonly used commercial gold standard for *in vitro* gene delivery. The structure-activity relationship between the lipidoids was further studied with respect to small variations in chemical structures and the resulting efficiency in DNA delivery *in vitro*. Since these lipidoids are easy to synthesize and do not require a co-lipid for efficient DNA delivery, they could offer an inexpensive but effective alternative to other commonly used commercial gene delivery carriers.

This Chapter 3 describes the synthesis of a combinatorial library of quaternized lipidoids and the evaluation of their abilities to facilitate in vitro DNA delivery. By formulating liposomes with a neutral helper lipid, 1, 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), the quaternized lipidoids can significantly enhance their gene transfection abilities due to the weaken quaternized lipidoid and DNA binding and the hexagonal-phase forming propensity of liposomes. We further optimized the

liposome composition and DNA dose for gene transfection and investigated the structure-activity relationships of the lipidoid library in DNA delivery.

ACKNOWLEDGEMENT

First, I would like to thank my advisor Dr. Qiaobing Xu, for all of his support and guidance over the past two years. His endless pursuit of science, diligence, and unbelievable creativity always inspired me. I would also like to thank my committee members, Dr. David Kaplan and Dr. Kyongbum Lee, for their continuously encouragement, endless support, and helpful insights. I thank Biomedical Engineering Department, especially Tissue Engineering Resource Center for providing outstanding facilities for my master's work and giving me the opportunity to experience a broader spectrum of research.

Moreover, I would like to thank the entire group in Prof. Xu's lab and 200 Boston Ave. crews. They taught me how to operate the equipment in the lab. Special thank to Dr. Ming Wang, Dr. Xiaoshu Dai, Dr. Guokui Qin, Mr. Roberto Elia, Mr. Tien Lee for their nutritious insights, comments and help.

In addition, I would like to express my gratitude to Prof. Maria Flytzani-Stephanopoulos and Dr. Jerry H. Meldon for their guidance, encouragement and support through the two years.

Last but not the least, I want to thank all my family and friends, especially my parents, my grandparents, my sister, my uncle, and my wife Yang Shu, for their support and love throughout my study at Tufts.

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CHAPTER 1 INTRODUCTION

1.1 Virus vectors

Gene therapy was first conceptualized in 1972, with the authors urging caution before commencing gene therapy studies in humans[1]. Today, acquired diseases such as cancer, cardiovascular disease, disorders of bones and joints, neurodegenerative disorders and infectious diseases are the subjects of most gene-therapy research[2-6]. One major step for gene therapy relies upon methods of introducing the desired genes into the proper cells. A perfect delivery vehicle depends on many important factors, as shown in Figure 1. Briefly, a perfect gene delivery vector must be efficient in delivery to the target cells and the introduced cells can be expressed with a high expression rate; it must have the ability to deliver to the specific target cell; it will not stimulate the immune response; it will not affects the dividing or non-dividing target cell, etc.

Viral vectors are one of the major efficient vehicles used to deliver the protein of interest encoding gene into target cells for the purpose of gene therapy. At present, five main classes of virus such as oncoretroviruses, lentiviruses, adenoviruses, adeno-associated viruses (AAVs) and herpes simplex-1 viruses (HSV-1s) can be transformed into gene delivery vehicles by replacing part of the genome of a virus with a therapeutic gene[7]. Because viruses evolved essentially as sophisticated gene delivery vehicles, such recombinant viral vectors are typically very efficient. Viral vectors have been used in the majority of gene delivery studies reported in the literature and of ongoing clinical trials[8]. Figure 2 shows a survey of gene transfer clinical trials originally from the Journal of Gene Medicine Clinical Trial Database. It indicates that most gene therapy

clinical trials are designed to treat cancer. Retrovirus and adenovirus vectors are the most popular viral vectors in gene transfer trials. However, most work has focused on the development of lentivirus vectors, which rely on active transport of the preintegration complex through the nucleopore by the culear import machinery of the target cell. The lentiviral strategy for nuclear targeting enables infection of non-dividing cell, an attractive attribute for a gene therapy vector[9].

However, in 1999, 18-year-old Jesse Gelsinger died as the result of volunteering for a gene therapy clinical trial for the partial deficiency of ornithine transcarbamylase (OTC), a liver enzyme that is required for the safe removal of excessive nitrogen from amino acids and proteins. OTC deficiency leads to an accumulation of ammonia in the bloodstream, which, in turn, causes an elevation of ammonium ions in the brain, leading to encephalopathy, brain damage and coma. He died of toxic shock after receiving the adenovirus vector carrying the transgene. The systemic delivery of the vector triggered a massive inflammatory response that led to disseminate intravascular coagulation, acute respiratory distress and multiorgan failure[10, 11]. In October and December 2002, the Necker Hospital in Paris announced that the two youngest boys enrolled into a gene therapy study for the treatment of X-linked severe combined immunodeficiency (X-SCID) had developed a leukemia-like disease[12]. These two incidents drew the researchers' attention on the two major obstacles to gene therapy and the dangers posed: immune reactions against the vectors and transgenes, and inappropriate insertion of vectors and transgenes that can cause mutations leading to cancer. Although viral gene therapy has high efficacy, it is plagued by serious safety risks, production and manufacturing challenges and other limitation including nucleic acid cargo capacity[8].

Thus, more work is needed to solve the safety concerns of viral delivery vectors.

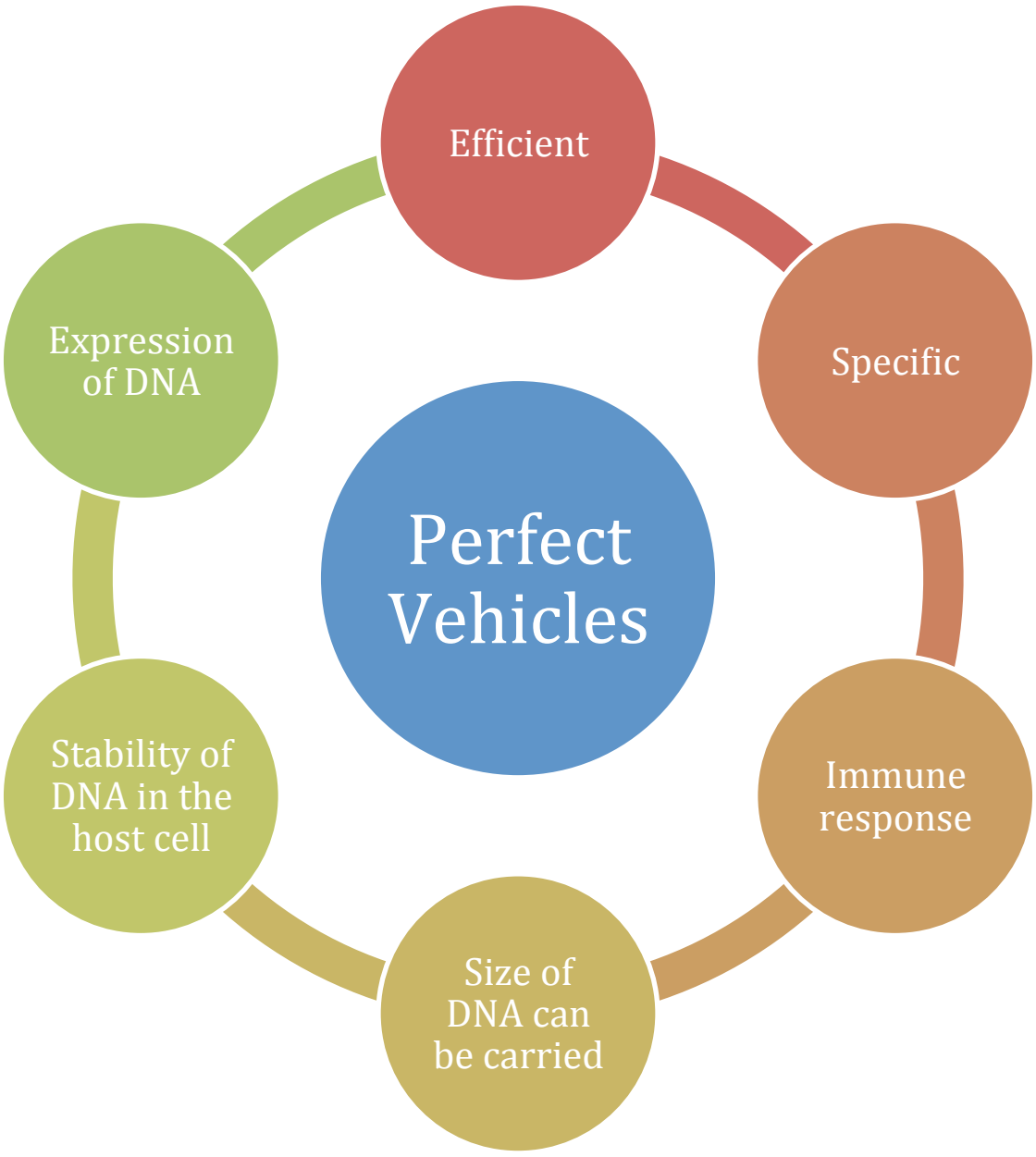


Figure 1. Factors composed a perfect delivery vector

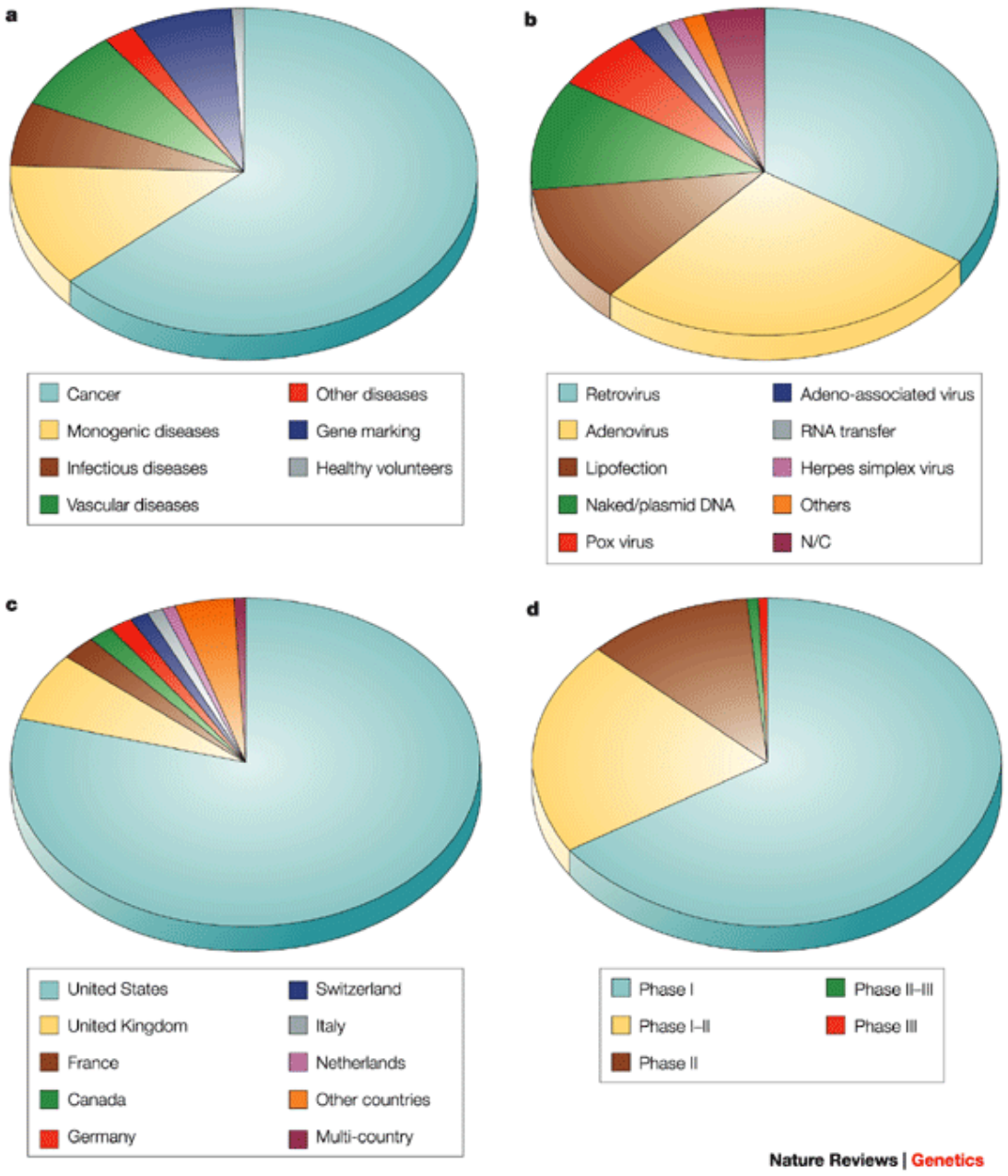


Figure 2. A survey of gene transfer clinical trials. -Kay M. A. *el at*. Nature Reviews Genetics. **2003**, *4*, 346-358

1.2 Non-virus vectors

In contrast to virus vectors, synthetic vectors provide opportunities for improved

Vector	Genetic material	Packaging capacity	Tropism	Inflammatory potential	Vector genome forms	Main limitations	Main ac
Enveloped							
Retrovirus	RNA	8 kb	Dividing cells only	Low	Integrated	Only transduces dividing cells; integration might induce oncogenesis in some applications	Persiste transfer dividing
Lentivirus	RNA	8 kb	Broad	Low	Integrated	Integration might induce oncogenesis in some applications	Persiste transfer most tis
HSV-1	dsDNA	40 kb* 150 kb‡	Strong for neurons	High	Episomal	Inflammatory; transient transgene expression in cells other than neurons	Large p capacity strong t neurons
Non-enveloped							
AAV	ssDNA	<5 kb	Broad, with the possible exception of haematopoietic cells	Low	Episomal (>90%) Integrated (<10%)	Small packaging capacity	Non-infl non-pat
Adenovirus	dsDNA	8 kb* 30 kb§	Broad	High	Episomal	Capsid mediates a potent inflammatory response	Extreme transdu most tis

*Replication defective. ‡Amplicon. §Helper dependent. AAV, adeno-associated viral vector; dsDNA, double-stranded DNA; HSV-1, herpes simplex virus-1; ssDN stranded DNA.

Table 1. The main groups of viral vectors-Kay M. A. *el at*. Nature Reviews Genetics. **2003**, *4*, 346-358

safety, greater flexibility and more facile manufacturing. In general, synthetic vectors are materials that electrostatically bind DNA or RNA, condense the genetic material into particles a few tens to several hundred nanometers in diameter (Figure. 3), protect the genes and mediate cellular entry. Various synthetic vectors, such as cationic polymers, calcium phosphate, (diethylamino) ether (DEAE)-dextran, and lipids, have been widely used fro in vitro and in vivo gene studies since 1960s.

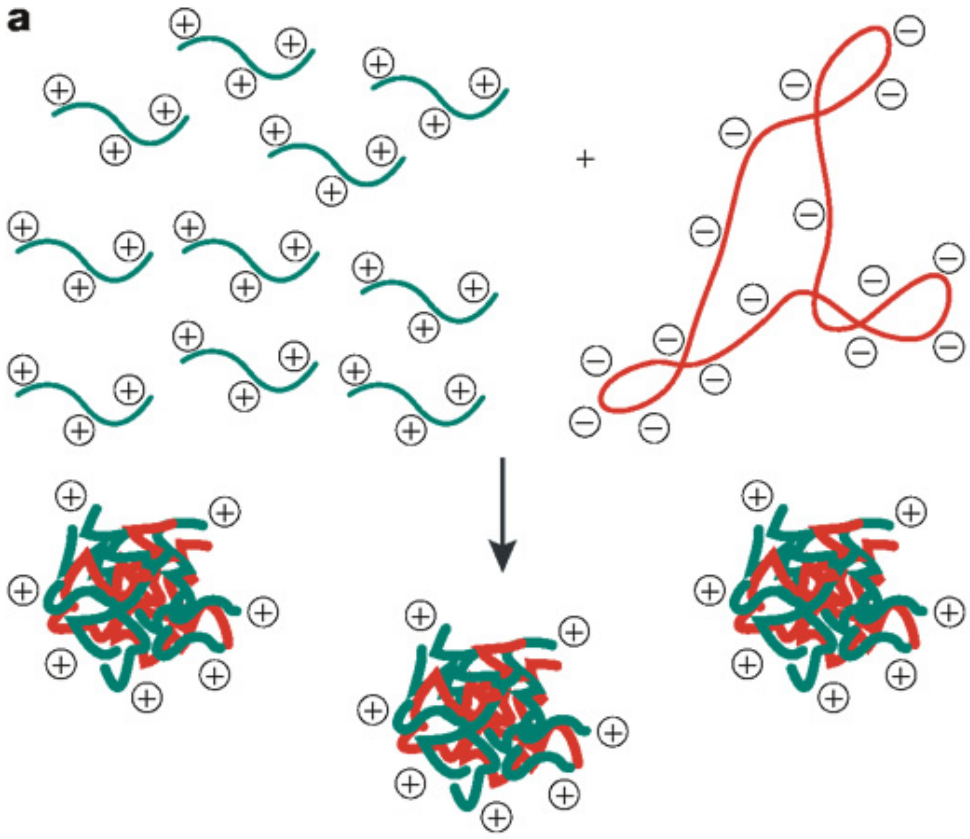


Figure 3. Polyplexes are formed by electrostatic interactions between polycations and DNA. - Stayton P. S. *el at.* Nature Reviews Drug Discovery. **2005**, *4*, 581-593

1.2.1 Cationic polymers for gene delivery

Polymeric systems for DNA delivery have been extensively studied in part due to their

well-defined chemistries and physical characteristics, and their high degree of molecular diversity that can be modified to fine-tune their target delivery properties[13]. Primary, secondary, tertiary and quaternary amines, as well as other positively charged groups such as amidines — can reside in the polymer backbone, in pendant groups or in grafted oligomers for the aim of effective DNA binding. There are three general types of cationic polymers used for gene delivery, including linear, branched, and spherical structures. Due to the flexibility of polymer chemistry, a specific polymer can be collectively altered to optimize one or more desired properties, such as delivery efficiency, stable formulation and biocompatibility, by regulation the molecular weight, polydispersity, side-chain composition and side-chain density, etc. As a result, cationic polymers have great potential for gene delivery.

Off-the-shelf polymers (Figure 4) have nonetheless been widely investigated and form the basis for much of the non-viral gene delivery literature. However, significant problems face these polymers. For example, polylysine was one of the first cationic polymers to be used for gene delivery. But due to their poor escape from the endocytic pathway, polylysine now seems unlikely to be applied for clinical trials with the relatively low delivery efficiency. Polyethylenimine (PEI) has also been a popular delivery vector since 1995[14]. Although it has a relatively high gene delivery activity, PEI has been hindered by its high cytotoxicity.

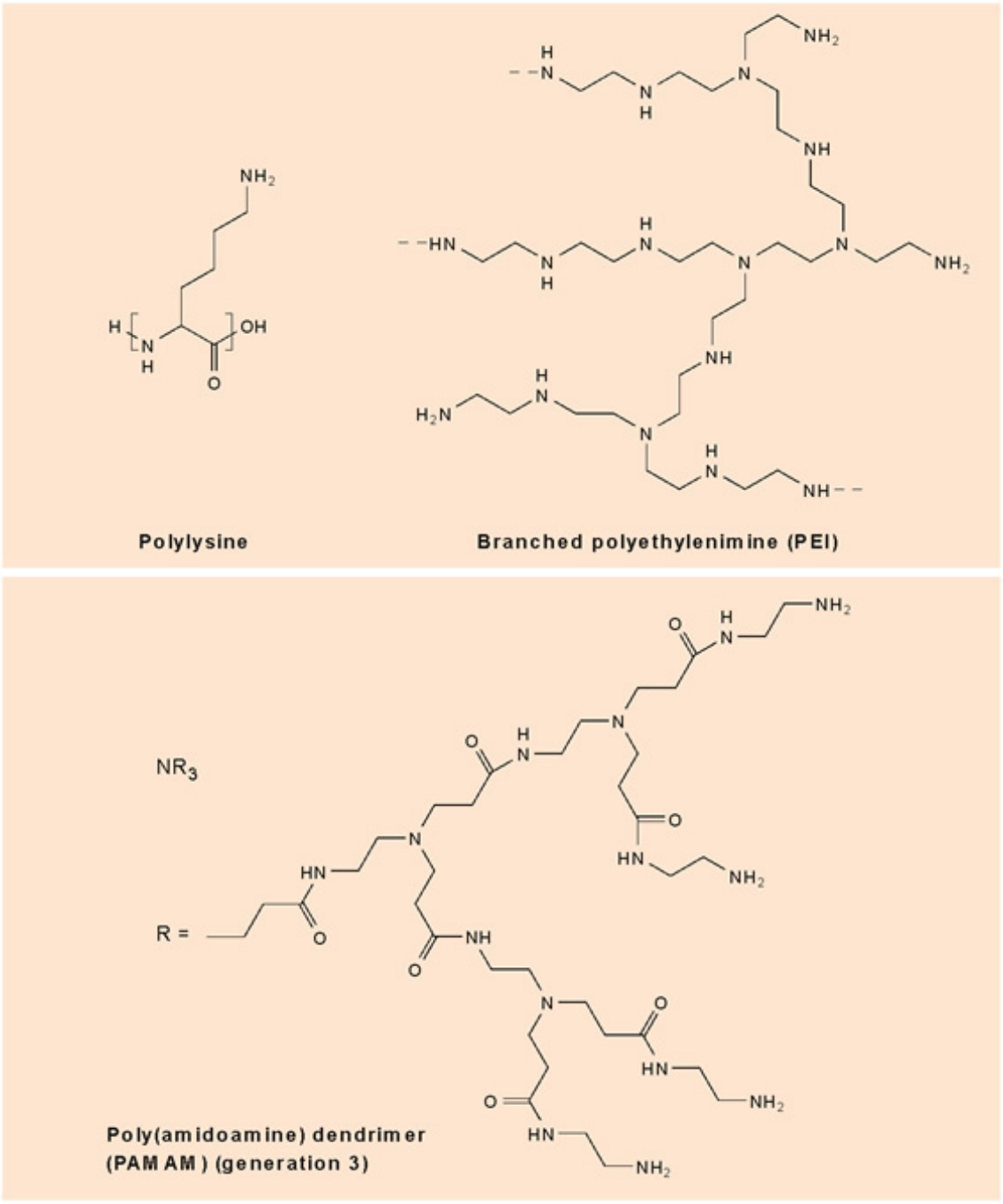


Figure 4. Structures of off-the-shelf gene delivery polymers In order to address the problems of the off-the-shelf polymers, many types of polymers have been specifically designed for gene delivery. In most cases, the polymers were designed to address a specific intracellular barrier (Figure. 5), such as stability, biocompatibility and endosomal

escape. Figure 6 shows some structures of novel designed polymers for gene delivery from Stayton's review. However, the delivery efficiency of polymers remains much lower than viral vectors. As a result, polymers are generally considered unacceptable for clinical applications.

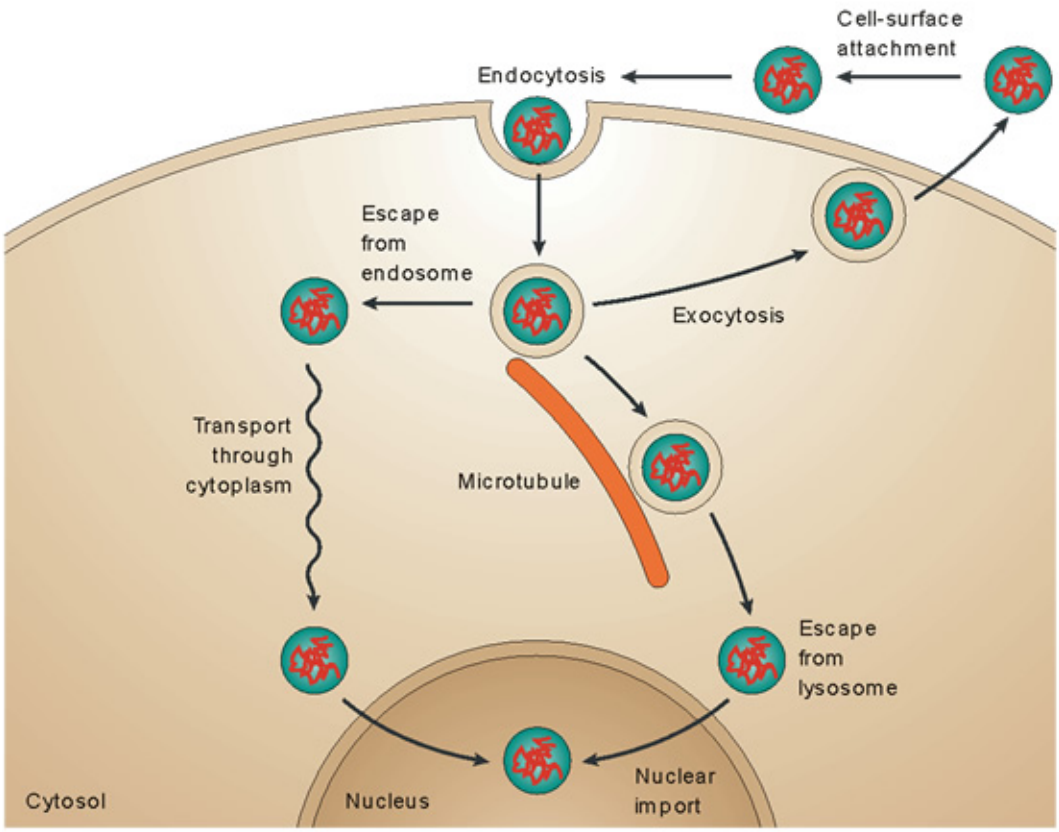


Figure 5. Barriers to intracellular trafficking of polyplexes. Polyplexes must attach to the cell surface, be internalized (by endocytosis), escape from endolysosomes, move through the cytoplasm toward the nucleus and cross the nuclear membrane. Alternative pathways exist for several of these steps. In addition, the polyplexes must unpackage — DNA must be released by the polymer — but where unpackaging occurs is not known. – Stayton *et al.* Nature Reviews Drug Discovery, **2005**, *4*, 581-593

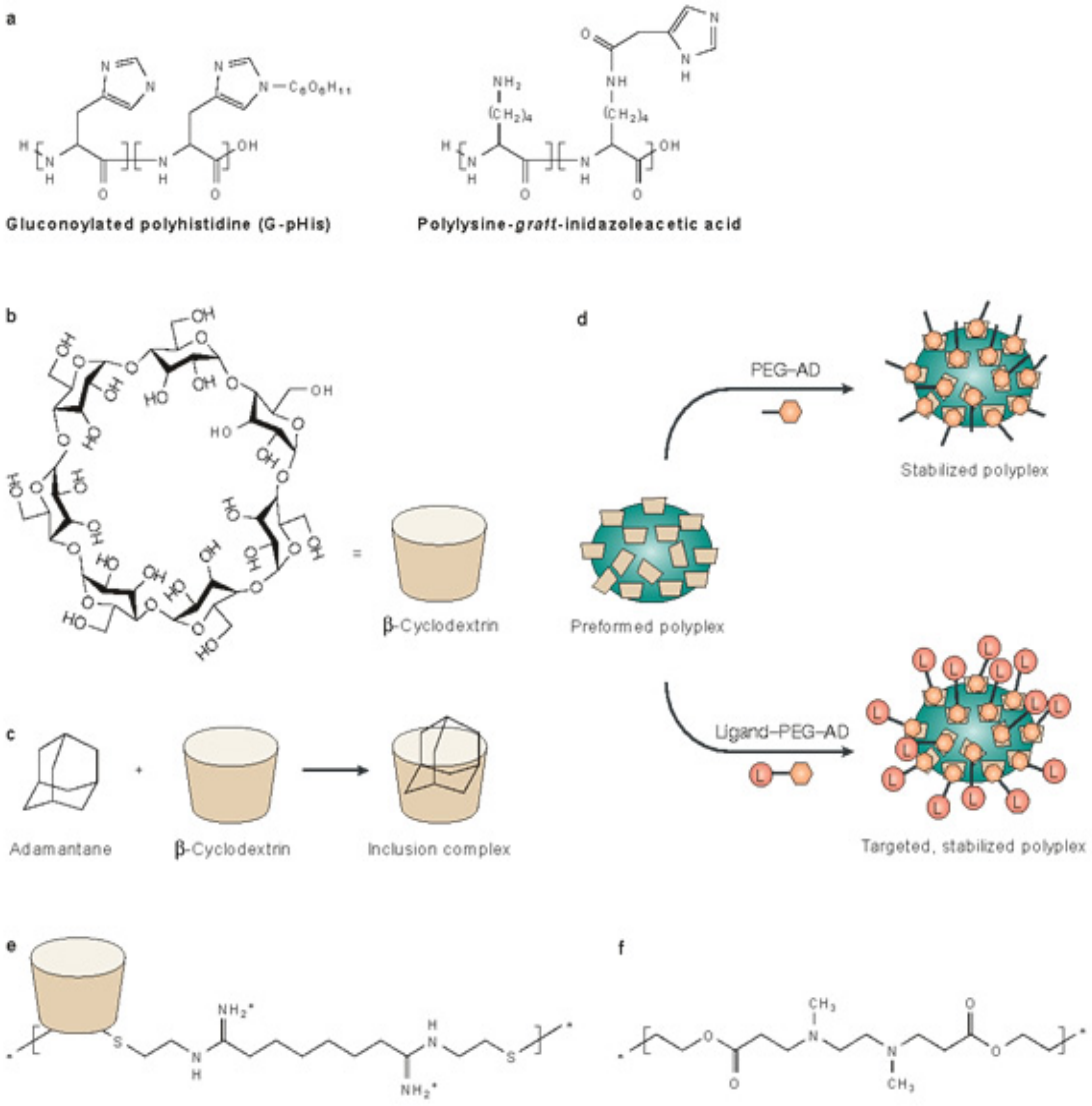


Figure 6. Structures of polymers designed for gene delivery. (a) Imidazole-containing polymers generated by conjugation of a sugar group to polyhistidine or histidylation of polylysine (b) The 'cup' of *β*-cyclodextrin (CD) has a hydrophobic interior and hydrophilic exterior surface. (c) *β*-CD is capable of binding non-polar molecules such as adamantane (AD) inside the cup to form inclusion complexes. (d) Polyplexes containing *β*-CD can be decorated with adamantane-terminated shielding groups such as polyehtylene glycol (PEG) to improve serum stability or with targeting ligands via

modification of the targeting group with adamantane. (e) Chemical structure of a *β*-CD-containing polymer. (f) Structure of a typical poly (*β*-amino ester)- Stayton *et al.* Nature Reviews Drug Discovery, **2005**, *4*, 581-593

1.2.2 Cationic lipids for gene delivery

Cationic lipid is another major class of non-viral gene delivery vectors, which are simple and quick to formulate, are not as biologically hazardous as viral vectors, are readily available commercially, and may be relatively easily adapted for specific applications[15, 16]. Cationic lipids have been developed for gene delivery since Felgner et al. first succeeded to deliver gene into cells by using unnatural diether-linked cationiclipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) in 1987[17]. Lipids are composed of three basic domains: a positive charged headgroup, a hydrophobic chain, and a linker that joins the polar and non-polar regions[18]. Figure 7 displays the three basic domains of 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP).

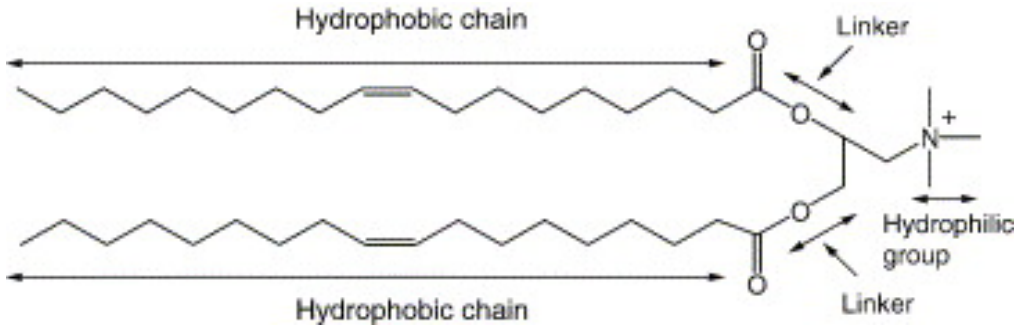


Figure 7. The structure of commonly used cationic lipid (DOTAP) for gene delivery.

The mechanism of gene delivery by cationic lipoplexes has now generally accepted that liposome mediated gene transfer proceeds primarily through endocytosis (Figure 8). The complexation of DNA with positively charged lipids or amphiphiles results from electrostatic interactions between the negatively charged phosphate backbone of DNA and positively aligned lipid. Unless a specific targeting ligand is provided on the lipoplexes, the binding of complex to the cell membrane is the result of a nonspecific ionic interaction between positive charge of lipids and negative charge of the cell membrane. Cellular uptake of lipid/DNA particle is the resulted from the endocytosis of the carrier[19]. Following the cellular uptake, in the early endosome, membrane destabilization results in phospholipid flip-flop reorganization. These phospholipids then diffuse into the lipoplex and interact with the cationic lipids leading to dissociation of nucleic acid from the particle and release into cytoplasm[20].

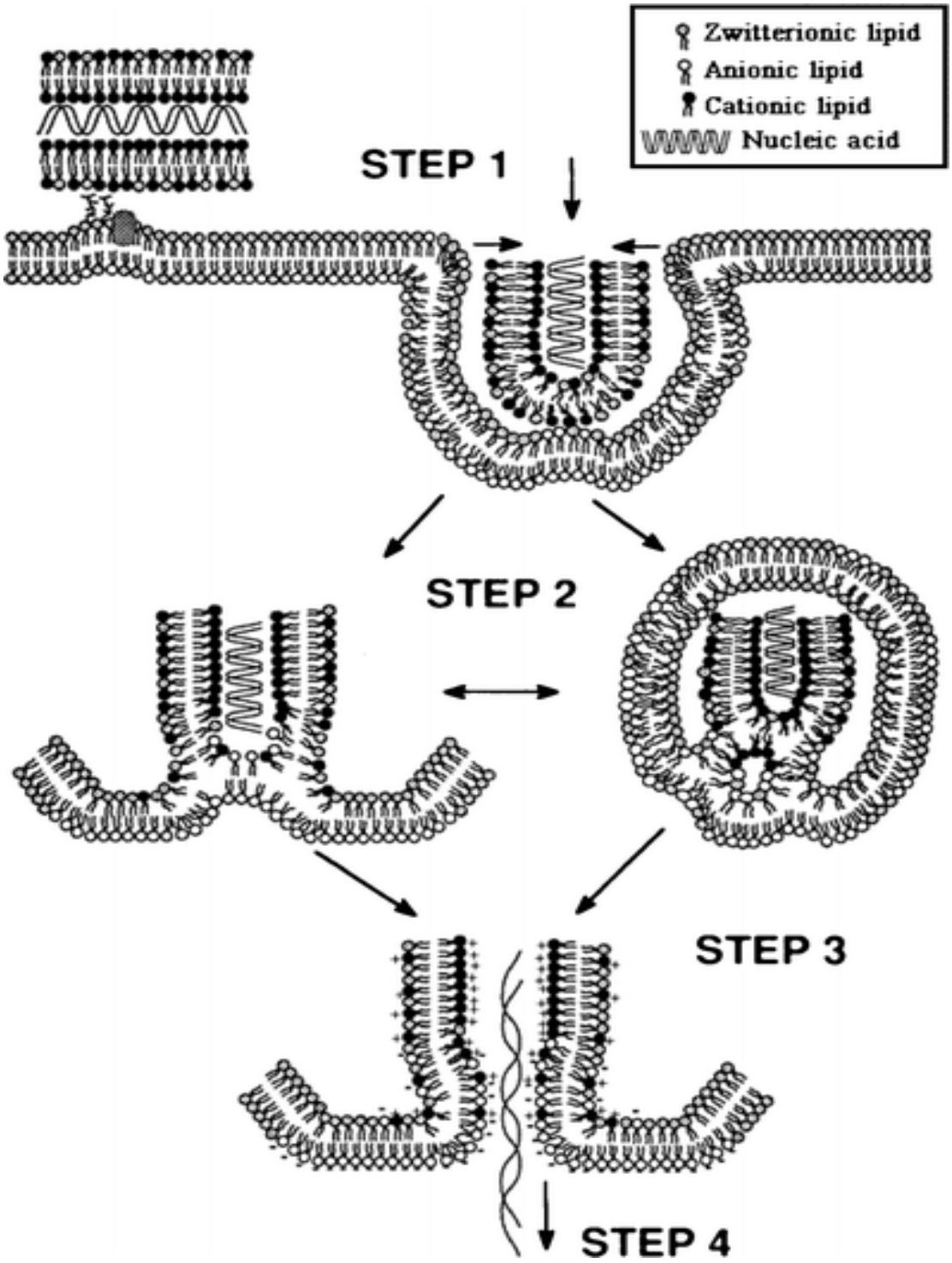


Figure 8. Mechanism of uptake and release of nucleic acid from the lipoplexes.

Szoka *et al*., Proc. Natl. Acad. Sci. **1996**, *93*,11493-11498

Active drug	Product name‡	Indications
Daunorubicin	DaunoXome	Kaposi's sarcoma
Doxurubicin	Mycet	Combinational therapy of recurrent breast cancer
Doxorubicin in PEG-liposomes	Doxil/Caelyx	Refractory Kaposi's sarcoma; ovarian cancer; recurrent breast cancer
Amphotericin B	AmBisome	Fungal infections
Cytarabine	DepoCyt	Lymphomatous meningitis
Vincristine	Onco TCS	Non-Hodgkin's lymphoma
Lurtotecan	NX211	Ovarian cancer
Nystatin	Nyotran	Topical antifungal agent
All-*trans* retinoic acid	Altragen	Acute promyelocytic leukaemia; non-Hodgkin's lymphoma; renal-cell carcinoma; Kaposi's sarcoma
Platinum compounds	Platar	Solid tumours
Annamycin		Doxorubicin-resistant tumours
E1A gene		Various tumours
DNA plasmid encoding HLA-B7 and α2 microglobulin	Allovectin-7	Metastatic melanoma
Liposomes for various drugs and diagnostic agents (lipoMASC)		Broad applications

Table 2 Liposomal drugs approved for clinical application or undergoing clinical evaluation

Liposomes are spherical, self-closed structures formed by one or several concentric lipid bilayers with an aqueous phase inside and between the lipid bilayers. The unique

structure of liposomes makes them a new generation of non-viral delivery vectors. For example, Liposomes can entrap water-soluble (hydrophilic) pharmaceutical agents in their internal water compartment and water-insoluble (hydrophobic) pharmaceuticals into the membrane. Moreover, liposomes provide a unique opportunity to deliver pharmaceuticals into cells or even inside individual cellular compartments. Furthermore, the size, charge and surface properties of liposomes can be easily changed simply by adding new ingredients to the lipid mixture before liposome preparation and/or by variation of preparation methods.

A number of researchers are developing new methods and systems in order to improve the drug encapsulation, retention, and stability of liposome both *in vitro* and *in vivo* environment[21, 22]. The clinical applications of liposomes are well known (Table 2). One of the drawbacks of the use of liposomes is the fast elimination from the blood and capture of the liposomal preparations by the cells of the reticulo-endothelial system, primarily in the liver. Numerous novel technics have been developed to reduce this problem [Figure. 9]. For examples, targeted liposomes with surface-attached ligands capable of recognizing and binding to cells of interest have been developed in order to enhance liposomal target delivery. Immunoglobulins (Ig) of the IgG class and their fragments are the most widely used targeting moieties for liposomes. Modified liposomes and antibodies maintain their integrity and targeting properties[22]. Long-circulating liposome grafted with a protective polymer such as PEG, which shields the liposome surface from the interaction with opsonizing proteins and therefore subsequent protects liposomes to have a long-circulating property[23]. In addition, both protective polymer and antibody can be modified to the liposome surface to make it a long-circulating

immunoliposome[24].

The initial success achieved with many liposome-based drugs has fuelled further clinical investigations. It would seem that liposomals drugs have a very promising future. However, considering the complicated synthesis of lipids and lower delivery efficiency *in vivo*, it is necessary to develop the lipid formulation in a pertinent way.

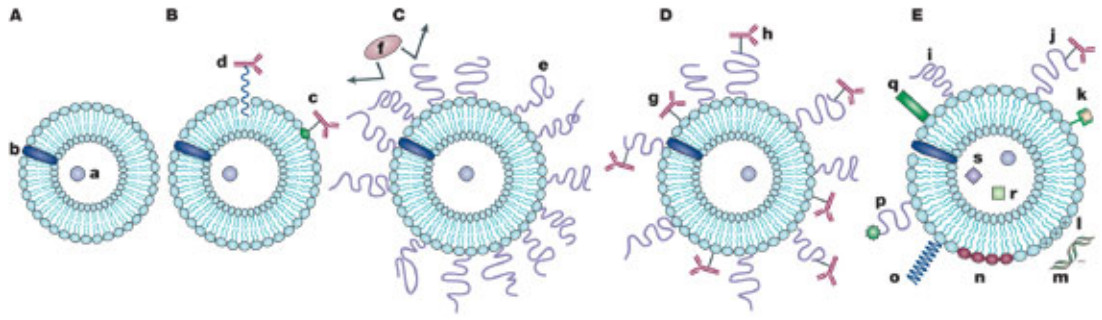


Figure 9. Evolution of liposomes. (**A**) Early traditional phospholipids 'plain' liposomes with water soluble drug (**a**) entrapped into the aqueous liposome interior, and water-insoluble drug (**b**) incorporated into the liposomal membrane (**B**) Antibody-targeted immunoliposome with antibody covalently coupled (**c**) to the reactive phospholipids in the membrane, or hydrophobically anchored (**d**) into the liposomal membrane after preliminary modification with a hydrophobic moiety. (**C**) Long-circulating liposome grafted with a protective polymer (**e**) such as PEG, which shields the liposome surface from the interaction with opsonizing proteins (**f**). (**D**) Long-circulating immunoliposome simultaneously bearing both protective polymer and antibody, which can be attached to the liposome surface (**g**) or, preferably, to the distal end of the grafted polymeric chain (**h**). (**E**) New-generation liposome, the surface of which can be modified (separately or simultaneously) by different ways. Among these modifications are: the attachment of

protective polymer (**i**) or protective polymer and targeting ligand, such as antibody (**j**); the attachment/incorporation of the diagnostic label (**k**); the incorporation of positively charged lipids (**l**) allowing for the complexation with DNA (**m**); the incorporation of stimuli-sensitive lipids (**n**); the attachment of stimuli-sensitive polymer (**o**); the attachment of cell-penetrating peptide (**p**); the incorporation of viral components (**q**). In addition to a drug, liposome can loaded with magnetic particles (**r**) for magnetic targeting and/or with colloidal gold or silver particles (**s**) for electron microscopy. - Torchilin V. P. Nature Reviews Drug Discovery, **2005**, *4*, 145-160

1.2.3 Lipidoids for gene delivery

Over the last thirty years, RNA as a translation template for protein synthesis has changed its activities within the cell. In particular, the significance of therapeutic meaning of RNA-mediated regulation of gene expression has attracted the researchers' interest. This technology, termed RNA interference (RNAi), is based on the ability of short duplexes of RNA, termed siRNA, to induce the specific cleavage of complementary mRNA, leading to a silencing of gene expression. RNAi has potential both for use as a tool capable of elucidating cellular mechanisms and as a therapeutic for treating disease at the genetic level[25].

Naked siRNA is the simplest option but has primarily been limited to diseases of the eye, since the eye is a relatively isolated tissue compartment, which provides advantages for utilization of siRNA[26]. For most other indications, a carrier is required to package and protect the molecules from serum nuclease and to selectively target the compounds to the desired tissue, which is the same as other gene delivery systems. The tissue and cellular pathway targeted in a particular disease determine the choice of an efficient RNAi

delivery strategy. In this case, it is probably unwise to assume a single type of delivery vehicle would have a good therapeutic effect on all diseases.

Recently, researchers at Massachusetts Institute of Technology have developed a class of lipid-like material that are nearly 100 times more efficient at delivery siRNA than previously studied lipid-based carries[27]. Lipidoids and lipids share many of the physicochemical properties. However, lipidoids require many fewer steps to synthesize and purify, which make rapid throughput combinatorial synthesis and rapid screening of potential drug delivery candidates possible. Figure 10 shows the synthesis of lipidoids for low dosing siRNA delivery. Low dosing is a key advantage of lipidoids over other RNAi delivery systems. It could reduce off-target target tissue toxicity and lower intracellular toxicity that arise from high levels of exogenous siRNA interfering with the endogenous RNAi processing machinery.

Mahon K. P. el at reported a library that have a mixture of lipid-like tails and feature appendages containing hydroxyl, carbamate, ether, or amine functional groups as well as variations in alkyl chain length and branching. Using a luciferase reporter system in HeLa cells, they studied the relationship between lipid chemical modification and delivery performance *in vitro[25]*.

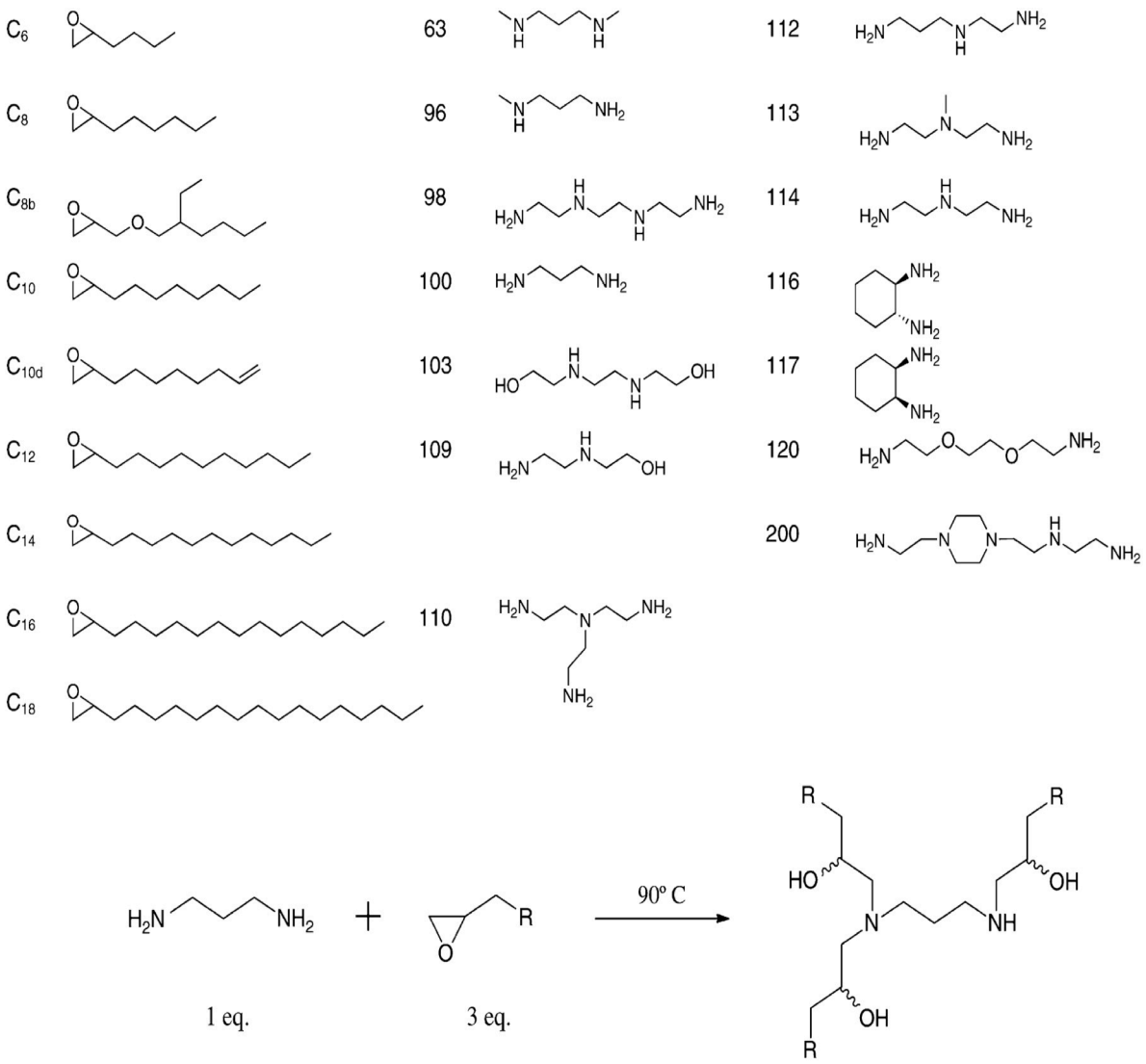


Figure 10. **Synthesis of Epoxide-Derived Lipidoid Library** (A) Epoxide terminated alkyl chains and amine-containing monomers were used in synthesis of combinatorial library, (B) addition of epoxides to amines by efficient ring-opening enables parallel synthesis of library members.

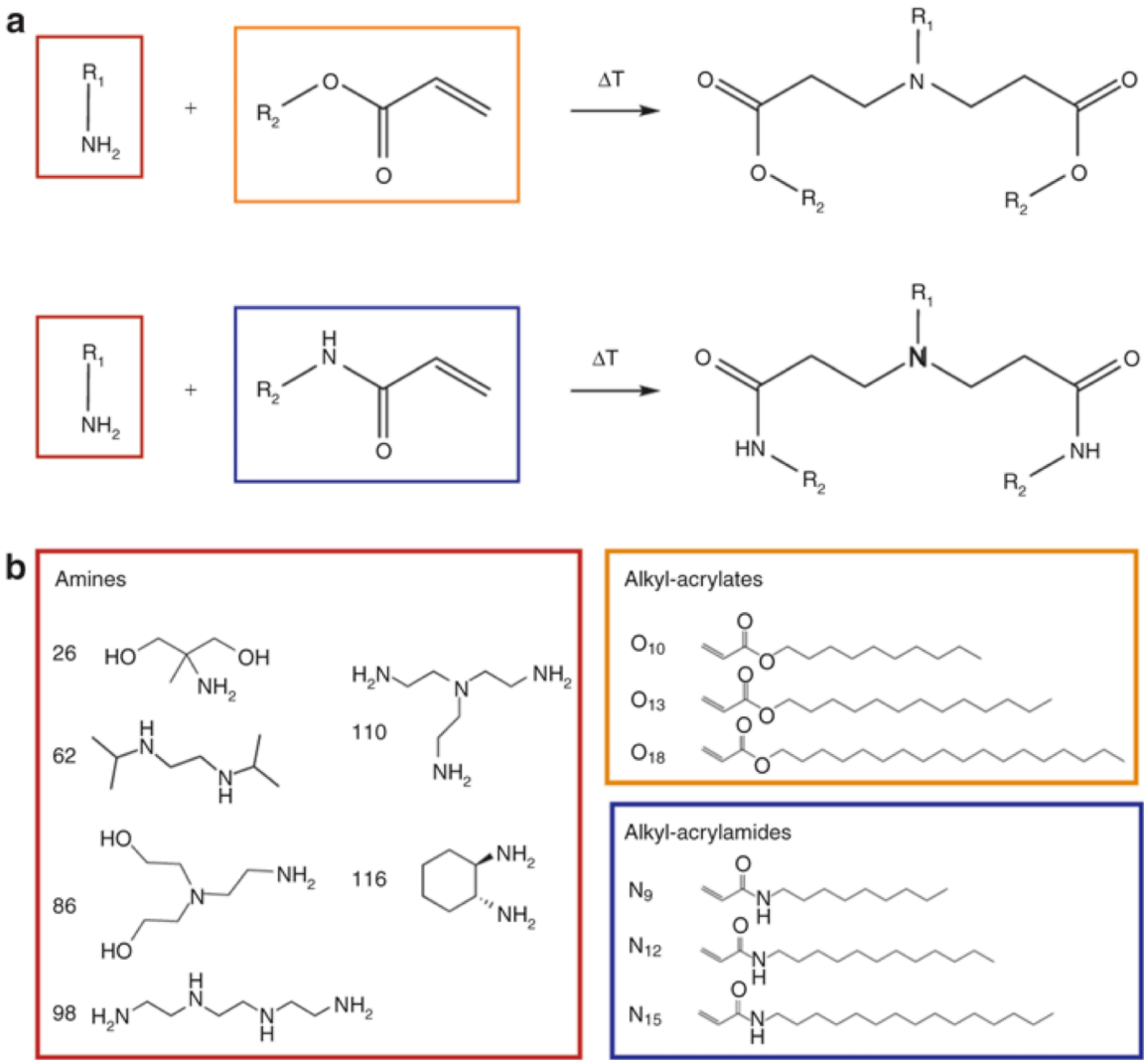


Figure 11. Lipidoid synthesis reaction and library. (**a**) A Michael addition reaction was used to synthesize lipidoids from the conjugate addition of amino molecules to alkyl-acrylate or alkyl-acrylamide molecules. (**b**) Six amines, three alkyl-acrylates, and three alkyl-acrylamides were used to create a library of 36 lipidoids. Lipidoids are named for the two molecules from which they were synthesized (*e.g.*, $26O_{10}$ or $98N_9$)- Whitehead K. A. *et al*, Molecular Therapy, **2001**, *19*, 1688–1694

In order to increase the material space available for development of siRNA delivery therapeutics, Whitehead K. A. et al examined the possibility of using binary

combinations of ionizable lipid-like materials (Figure 11) to synergistically achieve gene silencing. Interestingly, it was found that ineffective single lipid-like materials could be formulated together in a single delivery vehicle to induce near-complete knockdown of firefly luciferase and factor VII in HeLa cells and in mice, respectively[28]. Microscopy experiments (Figure 12) suggested that synergistic action resulted when combining materials that respectively mediated cellular uptake and endosomal escape, two important steps in the delivery process[28].

Based on lipidoid system, Nguyen D. N. et al studied the manipulation of TLR responses *in vitro* and *in vivo* by using lipidoid library for delivery of immunostimulatory RNA (isRNA) to TLR-expressing cells to drive innate and adaptive immune responses[29]. The engagement of TLRs 7 and 8 results in a characteristic type I interferon response (e.g., IFN-α), promotion of an antiviral state with induction of IFN-stimulated genes, and suppression of viral replication[30-33]. Effective lipidoid-isRNA nanoparticles, when tested in mice, stimulated strong IFN-α responses following subcutaneous injection, had robust antiviral activity that suppressed influenza virus replication, and enhanced antiovalbumin humoral and cell-mediated responses when used as a vaccine adjuvant[29].

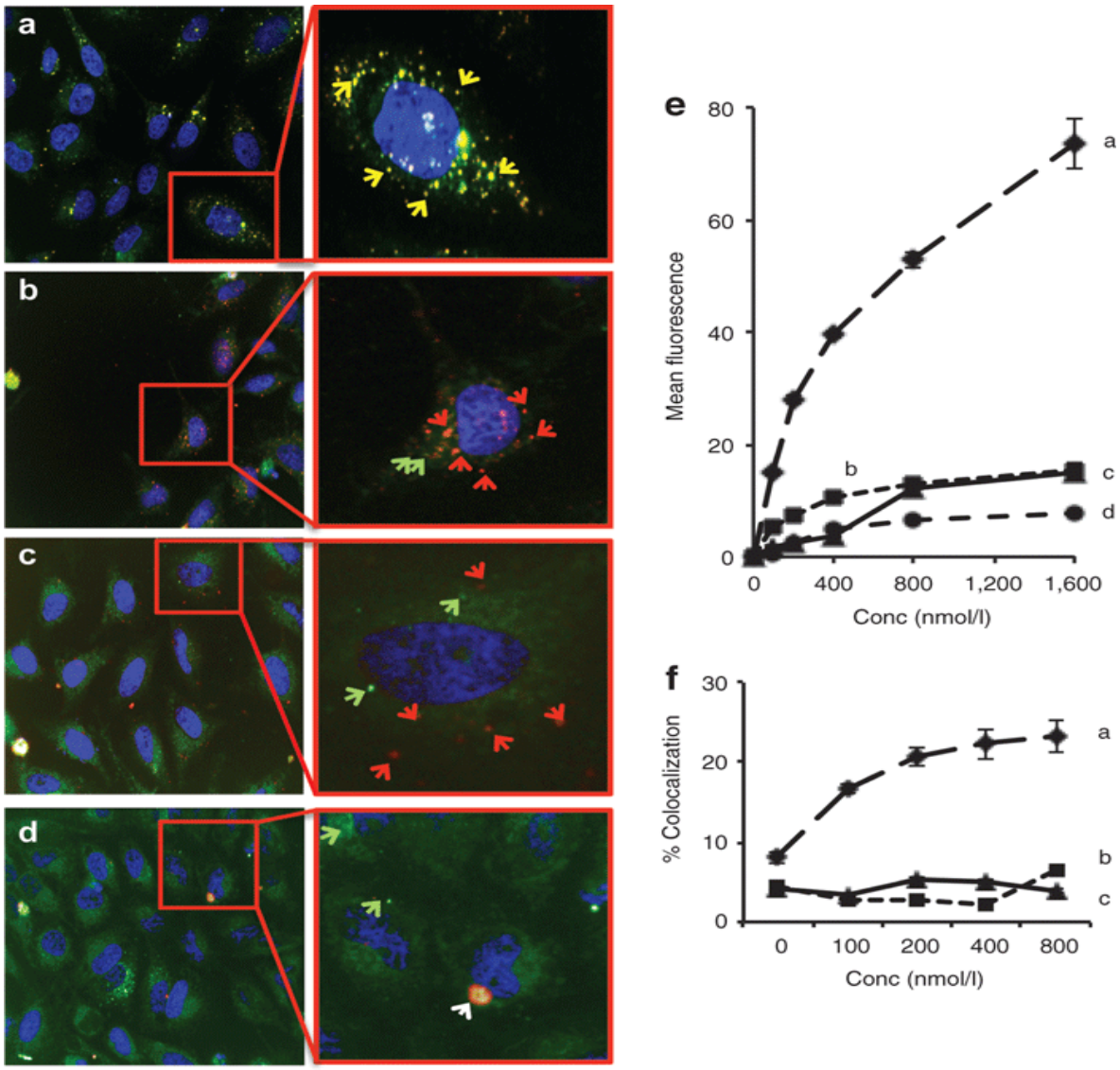


Figure 12. Synergistic 86N15–98O13 lipidoid combinations were uptaken into cells and avoided accumulation in the lysosomes. Confocal images were taken of the lipidoids (**a**) $86N_{15}$, (**b**) $86N_{15}$–$98O_{13}$ (weight fraction $98O_{13}$ = 0.5), (**c**) $86N_{15}$–$98O_{13}$ (weight fraction $98O_{13}$ = 0.75), and (**d**) $98O_{13}$ formulated into nanoparticles with Alexa-594-short interfering RNA (siRNA) and exposed to HeLa cells in the presence of Lysotracker Green. Representative images of the 400 nmol/l siRNA dose are shown. Inset-magnified images have been labeled with red and green arrows indicative of lipidoid and lysosomes, respectively. Yellow arrows show colocalization between the

lipidoid nanoparticles and the endosomes, while the white arrow in **d** shows aggregated nonspecific binding of lipidoid to plate. (**e**) Quantification of lipidoid internalization. (**f**) Quantification of internalized lipidoid nanoparticle colocalization with lysosomes. All quantification data presented was averaged from 20 different fields per well using Acapella software. -Whitehead K. A. *et al*, Molecular Therapy, **2001**, *19*, 1688–1694

CHAPTER 2 COMBINATORIAL LIBRARY OF LIPIDOIDS FOR IN VITRO DNA DELIVERY

2.1 Objective

Recently, a combinatorial library of lipid-like materials termed "lipidoids" was developed, and their utility as carriers for the delivery of siRNA and antisense oligonucleotides was described[27, 28, 34-36]. These lipidoids were prepared by reacting commercially available amines with lipophilic acrylates, acrylamides, or epoxide. The lipidoids have several advantages as a potential new class of nucleic acid delivery reagents: (i) the chemistry used to synthesize lipidoids is simple and economical, (ii) a library of structural diversity was already developed, (iii) a correlation between structure and function of delivery systems could be constructed from the large data sets accumulated from screening the library of lipidoids.

Utilizing lipidoids for DNA delivery, however, has not been previously reported. Lipidoids are structurally similar to many reported cationic lipids widely used for non-viral delivery of DNA[17, 37-39]. In this project, we evaluated the potential of lipidoids to facilitate DNA delivery *in vitro*. We demonstrated the capability of lipidoids synthesized from hydroxyl terminated diamines to deliver DNA efficiently into HeLa cells. We also studied structure-activity relationships of the lipidoids with regards to facilitating DNA delivery by designing a series of lipidoids with the same head and tail groups and differing linkers. We found that the lipidoids synthesized through amine and acrylamide addition reactions resulted in the most efficient DNA delivery compared to other lipidoids in the library. However, lipidoids synthesized with an ester linker were not stable and degraded completely in an hour via hydrolysis, hence losing the ability to

facilitate DNA delivery.

2.2 Materials and Methods

2.2.1 Synthesis of Lipidoids

Lipidoid library synthesis was performed and characterized as previously described[27, 35]. Amines and 1-aminoalkanes were purchased from Sigma-Aldrich (St. Louis, MO) and TCI America (Portland, OR). Acrylamides were synthesized by the drop-wise addition of acryloyl chloride to a solution of the appropriate 1-aminoalkane in dichloromethane in an ice-bath under an inert atmosphere. Lipidoids were synthesized by conjugation addition of amines to acrylamides, acrylates, or epoxides. These reactions were performed in 5-mL Teflon-lined glass screw-top vials. For lipioids prepared from acrylamides, the number of equivalents of acrylamide was equal to maximum number of conjugate additions possible for each amine (2 for primary amines). The reactions were performed on a 200 mg scale (of amine). The mixture was stirred at 90°C for 7 days. After cooling, the lipid mixtures were used without purification unless otherwise specified. Representative library members were characterized by thin layer chromatography, IR, NMR, and mass spectroscopy.

2.2.2 In Vitro DNA Transfection

HeLa cells were obtained from ATCC (Manassas, VA) and cultured in phenol red-free DMEM supplemented with 10% fetal bovine serum and 100 units/ml of penicillin/streptomycin at 37°C and 5% CO_2. All cell culture reagents were purchased

from Invitrogen Corporation (Carlsbad, CA) unless otherwise noted. pCMV *β*-gal DNA was also purchase from Elim Biopharmaceuticals, Inc (Hayward, CA). To facilitate screening throughput, lipidoid-DNA complexes were formed by simple mixing of lipidoid-DNA solutions in 50 mM sodium acetate buffer solution pH 5.5 in microtiter plates. For transfection in 96-well plates, HeLa cells were seeded (10,000 cells per well) into each well of an opaque white 96-well plate and allowed to attach overnight. Cells were transfected with 200 ng of DNA (per well) complexed with lipidoids at fixed lipidoid/DNA P/N ratio of 5:1. The lipidoids were added to the DNA solutions, and then incubated for 10 min at room temperature to allow for complex formation. The lipidoid/DNA solution was immediately added to cells in each well. After transfection, cells were incubated for 24 hrs at 37°C and 5% CO_2 before analyzing for *β*-gal protein expression. Control experiments were also performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), as described by the vendor. Transfection was performed in quadruplicate.

2.2.3 *β*-gal Activity of Cell Culture

β-gal activity assay is described by the developed protocol with slight modification[40]. Briefly, after the transfection over 24 hours, all growth medium in the 96-well plate was removed by vacuum aspiration. 100 mL PBS was added into each well to wash the cell for 5 min (×2) on ice. 50 ml triton (Sigma-Aldrich, St. Louis, MO) solution (0.5% in PBS) was added into each well right after removal of PB. The plate was then left on the ice for 10 min. Fifty mL of o-nitrophenyl-*β*-D-galactoside (ONPG) (Sigma-Aldrich, St. Louis, MO) solution (4 mg/mL in z-buffer) was added into each well by multi-channel

pipette.

The recipe of Z buffer (50 mL):

- 0.80 g $Na_2HPO_4 \cdot 7H_2O$ (0.06 M)
- 0.28 g $NaH_2PO_4 \cdot H_2O$ (0.04 M)
- 0.5 mL 1M KCl (0.01 M)
- 0.05 mL 1M $MgSO_4$ (0.001 M)
- 0.135 mL b-mercaptoethanol (BME) (0.05 M)
- Adjusting the pH to 7.0
- Stored at 4°C

The plate was incubated for 15 min at 37°C. The concentration of *β*-gal was determined by measuring the absorbance at 409 nm.

2.2.4 DNA binding Assay

PicoGreen assay was performed as previously described[41]. Briefly, in 96-well plate, 50 mL/well of lipidoid solutions at 1 mg/mL in NaOAc buffer were added to 50 mL/well of DNA (60 mg/mL in NaOAc buffer). The solutions were mixed vigorously and allowed to sit undisturbed for 5 min to allow for lipidoid-DNA complex formation. Then, 100 mL/well of PicoGreen working solution (Invitrogen,Carlsbad, CA) was added. PicoGreen working solution was prepared by diluting 52.8 mL of the purchased stock into 9.9 mL NaOAc buffer. After 5 min, 30 mL/well of lipidoid-DNA-PicoGreen solution was added to 200 mL/well of DMEM media in a black 96-well polystyrene plate. The plate fluorescence was then measured on a SpectraMax® M2 Multi-Mode Microplate Reader

(Molecular Devices, Inc. California, USA) at excitation of 485 nm and emission of 535 nm. The relative fluorescence (*RF*) was calculated by the following relationship:

$$RF=(F_{sample}-F_{blank})/(F_{DNA}-F_{blank})$$

where F_{sample} is the fluorescence of the lipidoid-DNA-PicoGreen sample, F_{blank} is the fluorescence of a sample with lipidoid or DNA (only PicoGreen), and F_{DNA} is the fluorescence of a sample with DNA-PicoGreen without lipidoid. The DNA encapsulation is one minus RF.

2.2.5 Transmission Electron Microscopy (TEM)

Lipidoid/DNA complexes were prepared with the same protocols as *in vitro* experiments (5:1 P/N ratio of total lipidoid to DNA) Droplets of the sample (5ml) were applied to hydrophilized carbon-covered copper grids (300 meshes) for 30 min. The sample was subsequently rinsed with contrasting material (1% uranyl acetate at pH 4.5). The remaining stain solution was removed with a filter paper and air-dried. TEM microstructure was determined using a Tecnai FEG TEM (FEI Tecnai 12 Spirit Bio-twin, FEI Company, Hillsboro, OR) operating at 80 kV.

2.2.6 Fourier Transform Infrared Spectroscopy (FTIR)

The structural characteristics were observed using FTIR as previously reported by Hu *et al*[42, 43]. Lipidoid/DNA nanoparticles were prepared as described in TEM section. Before the measurement, the lipidoid/DNA solution was dropped onto the detector stage, blowing with the air gun at room temperature until all the dissolvent evaporated.

Normalization of the curve was established through the protocol described in Hu's paper**.**

2.3 Results and Discussion

2.3.1 Screening of Lipidoid library

Lipidoids were prepared by heating commercially available amines with lipophilic acrylamides, acrylates, or epoxides without solvent or catalysts, according to our previous reported methods[27, 35]. The simplicity of these reactions allowed us to build a structurally diverse library of lipidoids by varying the types of amines, and the lengths and types (acrylamide/acrylate/epoxide) of tails[27, 35]. The resulting crude products were directly used for *in vitro* delivery of DNA.

In order to facilitate rapid throughput library screening, plasmid DNA encoding *β*-galactosidase (*β*-gal) was employed as the reporter gene. The *β*-gal enzymatic assay was carried out to measure the level of *β*-gal expression. HeLa cells, in their exponential growth phase, were seeded at 10,000 cells per well into 96-well plates to ~80% confluency the day before transfection. The library of crude lipidoids was initially screened by delivering lipidoid/DNA complex to the HeLa cells in 96-well plates. The screening results showed most lipidoids were not effective in facilitating DNA delivery, with transfection efficiencies lower than the leading transfection reagent, Lipofectamine 2000® (data not shown). However, we observed the crude lipidoids derived from amine **86** (N,N-Bis(2-hydroxyethyl)ethylene diamine) and amine **87** (N-(3-aminopropyl)diethaneamine) (Scheme 1, A) generally showed relatively high efficiency in DNA delivery, sometimes equal or higher than Lipofectamine. This observation led us to synthesize a refined library of purified lipidoids from the reaction of amines (**86** and

87) and different types and lengths of tails (acrylamide, acrylate and epoxide), shown in **scheme 1A.**

The lipidoids with different tail lengths were purified from the crude reaction mixtures by column chromatography and tested for their ability to deliver *β*-gal DNA to HeLa cells. The screening results showed that most of the pure lipidoids in the refined library supported DNA delivery into the HeLa cell with expression of *β*-gal (Figure 13); however the efficiency varied depending on the lipidoid structure. Lipidoids derived from amine **86** and **87** with two medium-length tails (e.g., C14 or C15 for acrylamide and acrylate, respectively, and C14 or C16 for epoxide) displayed high transfection efficiencies, in some cases higher than Lipofectamine 2000®. We observed that the linkers between tail and head groups of the lipidoids affect the efficiency in facilitating DNA delivery. The lipidoids with amide linkers displayed substantially higher delivery efficiency than those with ester and hydroxyl groups. The delivery efficiency also varied with the amine molecules utilized for lipidoids synthesis, for example, the acrylamide and epoxide conjugated with amine **87** (14N-87 and 16C-87) delivered DNA more efficiently than those conjugated with amine **86** (14N-86 and 16C-86), while for the lipidoids synthesized from acrylate, the reverse results were observed (Figure 13).

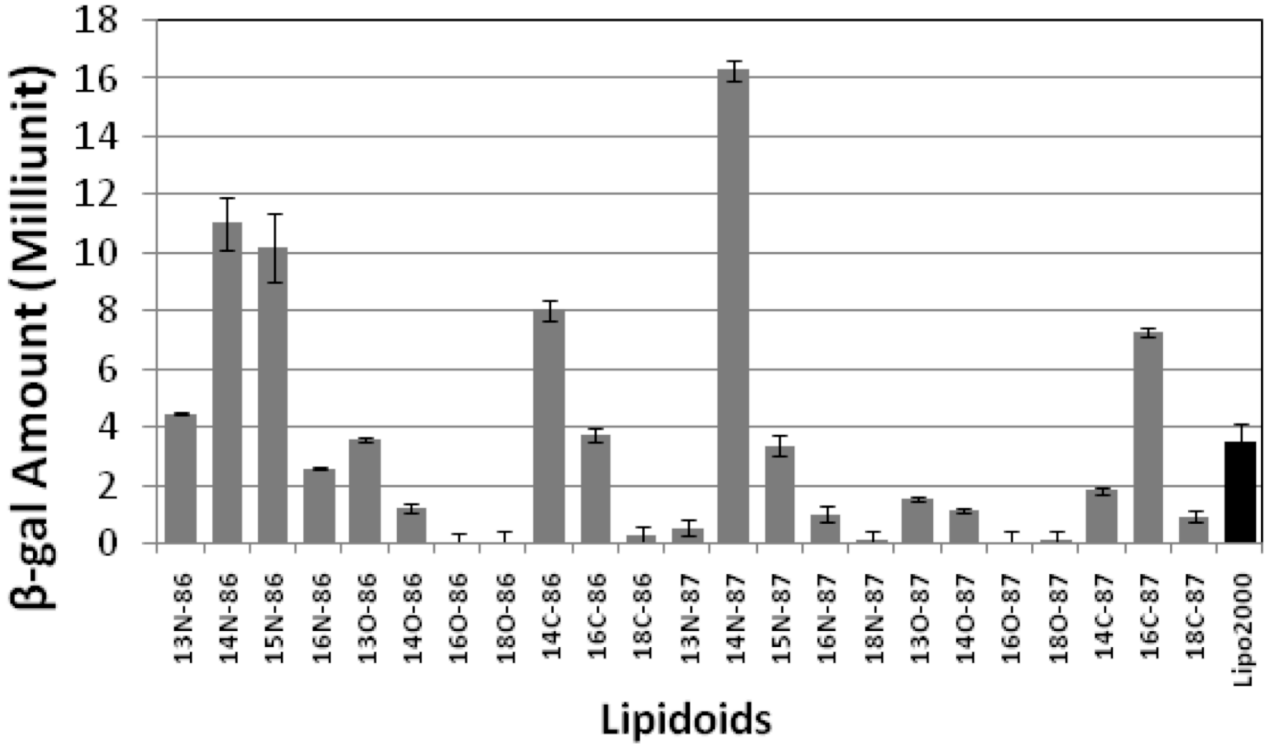
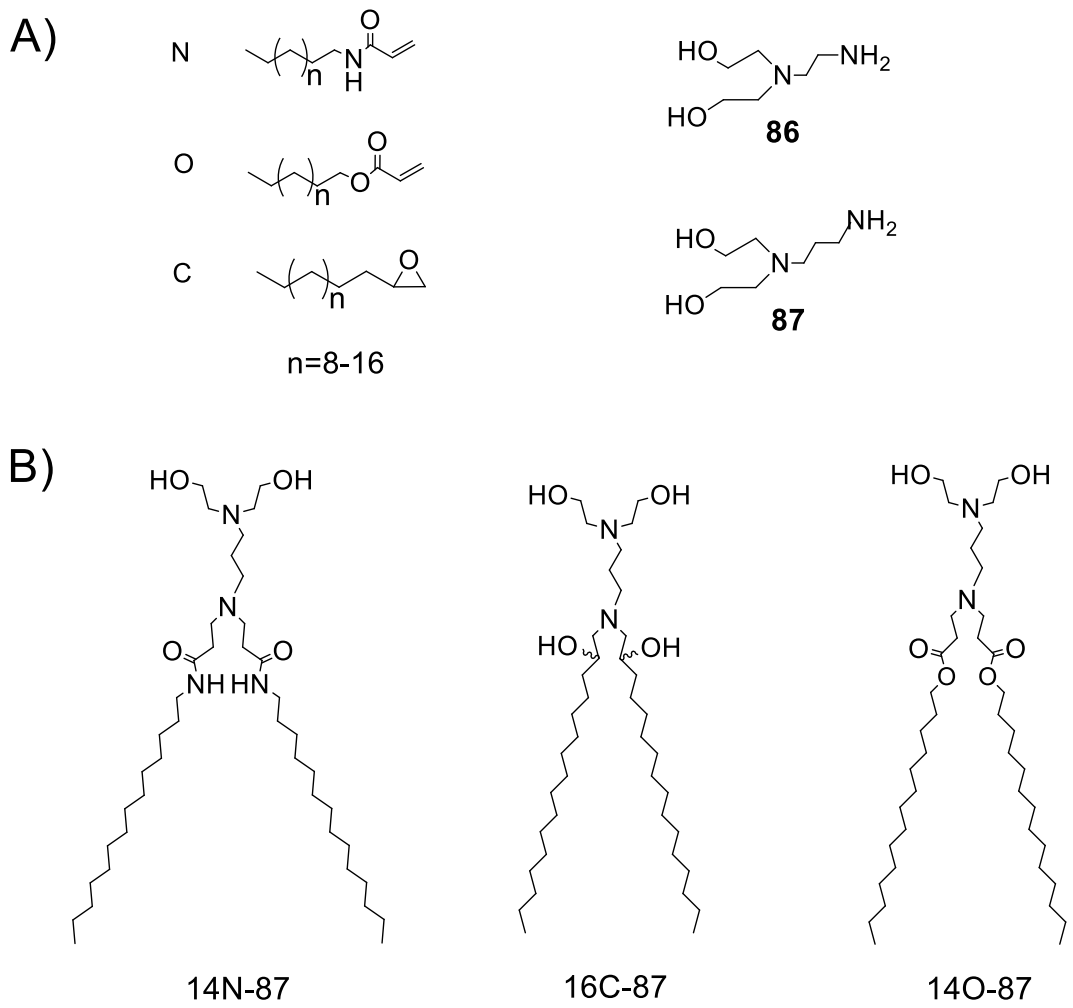


Figure 13. Initial *in vitro* screening of lipidoids for DNA delivery. Lipidoids were screened by delivering plasmid DNA encoding *β*-gal into HeLa cells. Relative *β*-gal expression level was determined by assaying the enzyme activity according to reported methods.



Scheme 1. Combinatorial synthesis of lipidoids for DNA delivery. (A) Alkyl-acrylamide, alkyl-acrylate, alkyl-epoxide, and amine molecules used for the library synthesis; (B) Chemical structures of the selected lipidoids with high DNA delivery efficiency. Lipidoids are named as follows: (carbon numbers of tail) (acrylate, acrylamide, or epoxide)-(amine number)

2.3.2 Effect of Positive to Negative (P/N) ratios on *β*-gal activity

To further investigate the structure-activity relationship of these lipidoids for DNA delivery, we selected lipidoids prepared from the reaction of amine **87** with acrylamide (14N-87), epoxide (16C-87), and acrylate (14O-87) (Scheme 1B), which displayed high delivery efficiency. DNA was then complexed with these lipidoids (immediately dissolved in sodium acetate buffer (50 mM, pH = 5.5) before use) in different weight ratios for delivery. The results showed the lipidoid to DNA ratios (w/w) affected the delivery efficiency and the resulting *β*-gal activity. These lipidoids have a Mw around 680 Da and two positive charges; a single base pair of DNA has a Mw around 660 Da and two negative charges. The weight/weight (w/w) ratio of the lipidoid/DNA has a similar value to the molar ratio and positive/negative (P/N) charge ratio. For all lipidoids selected, *β*-gal enzyme activity was detectable at the lipidoid/DNA ratio of 1:1 (Figure 14). The delivery efficiency was enhanced by an increasing ratio. For 14N-87 and 14O-87, the transfection efficiency increased to the highest level at a lipidoid/DNA ratio of 5:1 and slowly declined by further increasing the ratio. Lipidoid 16C-87, on the other hand, enhanced its transfection efficiency until the lipidoid/DNA ratio increased to 15:1. At the dose level used in these experiments, no obvious cytotoxicity was observed from the delivery reagents used. Under the same conditions, the 14N-87 showed higher tranfection efficiency compared with 14O-87 and 16C-87.

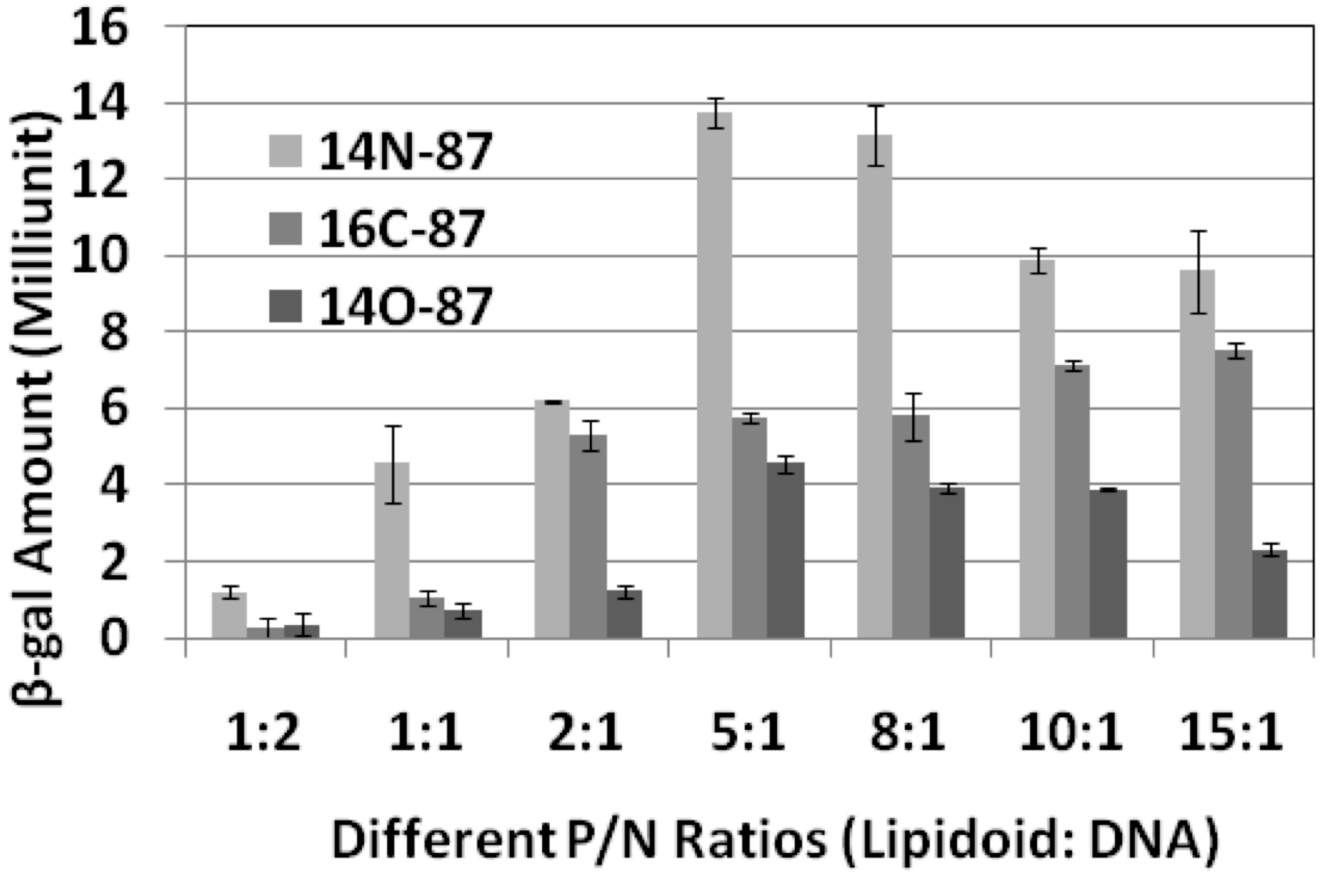


Figure 14. The relationship of positive to negative charge (P/N) ratios of lipidoids/DNA and DNA delivery efficiency. Lipidoids 14C-87, 16C-97, and 14O-87 were selected according to initial screening results. The

2.3.3 Morphology of Lipidoid/DNA complexes

It is known that the transfection efficiency of a given lipid-DNA complex depends on its structure and physicochemical properties[44]. We used negative-staining transmission electron microscopy (TEM) to study the size and morphology of the lipidoid-DNA complexes and to illustrate a structure-function correlation of lipidoids with biological activity. The 14N-87 was able to efficiently self-assemble with DNA through electrostatic interactions and form condensed multilamellar structure nanocomplexes (~100 nm) with DNA intercalated between the lipid bilayers (Figure 15). In contrast, 14O-87 and 16C-87 tended to form loose "spaghetti and meatball-like structures"[37]. The higher transfection efficiency of DNA delivery by 14N-87 may be attributed to its higher efficiency in nanoparticle formation compared with 14O-87 and 16C-87.

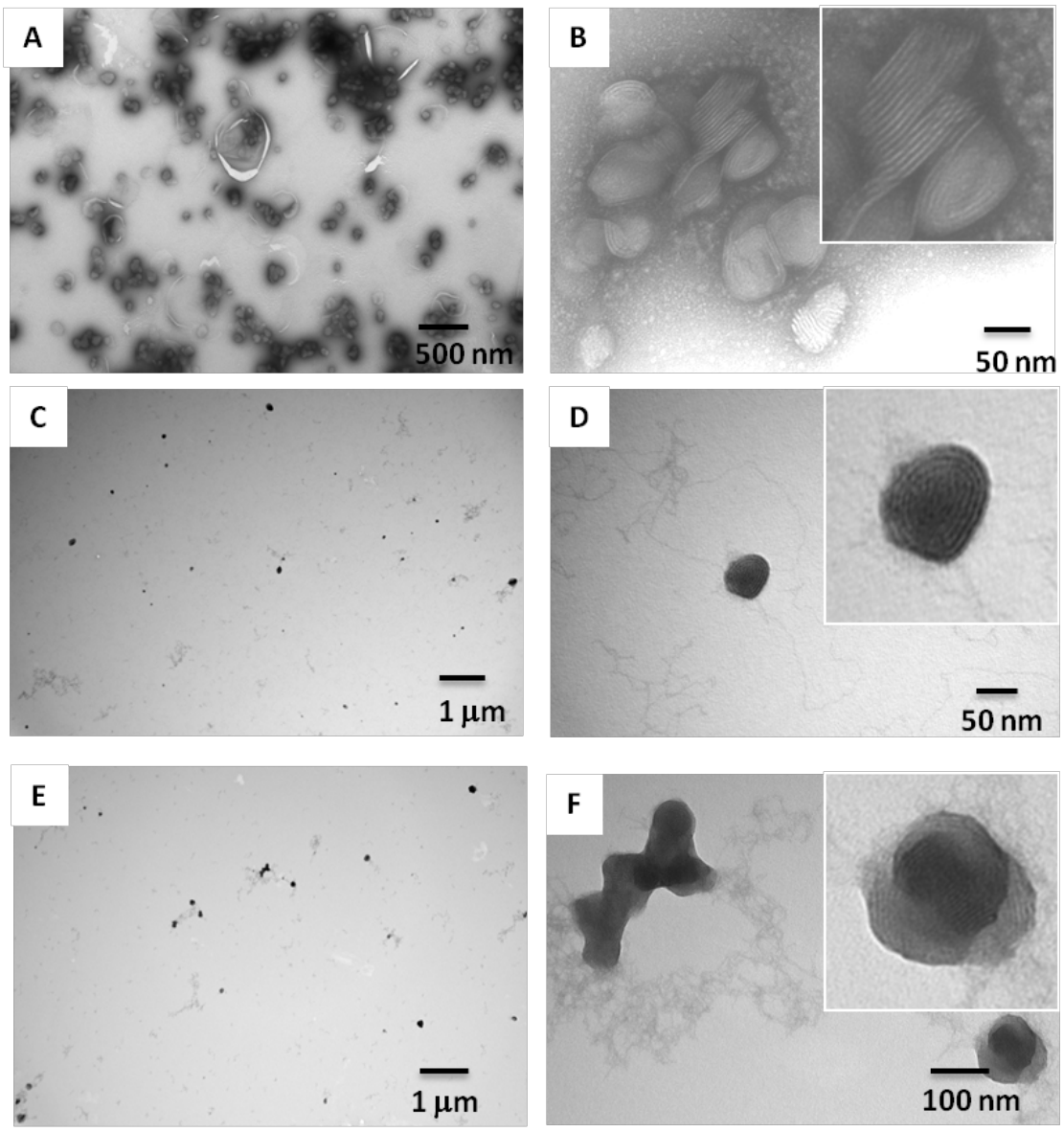


Figure 15. Negative staining TEM images of lipidoids/DNA (P/N=5:1) nanoparticles. A and B: 14N-87/DNA complex; C and D: 16C-87/DNA complexes; E and F: 14O-87 /DNA complexes.

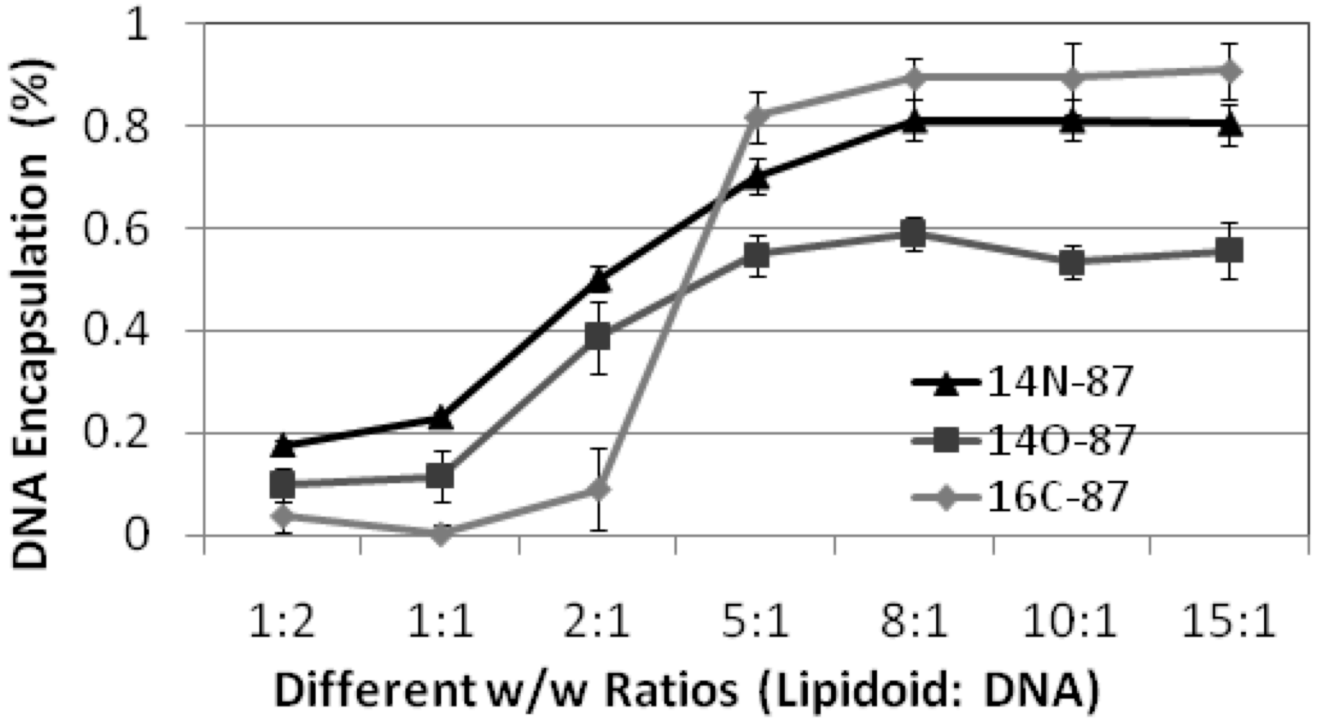


Figure 16. The Picogreen assay of the lipidoids (14N-87, 16C-87 and 14O-87)/β-gal encoding DNA complexes in different ratios.

2.3.4 PicoGreen Assay for DNA encapsulation

We next determined the efficiency of DNA encapsulation by different lipidoids at different P/N ratios using PicoGreen assay (Figure 16). At a low P/N ratio (<5), the DNA encapsulation percentage was low for all three lipidoids, which led to the observed low transfection efficiency (Figure 14). All three lipidoids showed increased DNA encapsulation with an increase of the P/N ratio. 14N-87 and 16C-87 had a maxium percentage of encapsulation of 80-90%, while 14O-87 reached 60%, which may explain why DNA delivery by 14N-87 and 16C-87 were more efficient than 14O-87. Although lipidoids 14N-87 and 16C-87 displayed similar binding abilities with DNA in ratios above 5:1, 14N-87 was generally more efficient in mediating DNA delivery than 16C-87. This was probably due to 16C-87 and DNA forming a large amount of flocculent nanostructures, while 14N-87 and DNA formed condensed individual multilamellar nanoparticles (Figure 15).

2.3.5 Hydrolysis of Lipidoid

We noticed that 14O-87 lost their capability in DNA delivery when the as-prepared solution was stored for a certain period of time (>2hrs), a phenomenon not observed with the other two lipidoids, 14N-87 and16C-87. No successful DNA delivery was observed using the stored 14O-87 compared with the freshly prepared sample (Figure 17). We hypothesize that the loss of ability to mediate DNA delivery is ascribed to the hydrolysis of the ester linker in 14O-87. This was confirmed by FTIR analysis of fresh and stored 14O-87 solutions (Figure 18). The peak at 1720 cm^{-1} assigned to the ester bond in 14O-87 weakened after 1 h in a sodium acetate solution and a new peak at 1680 cm^{-1} appeared.

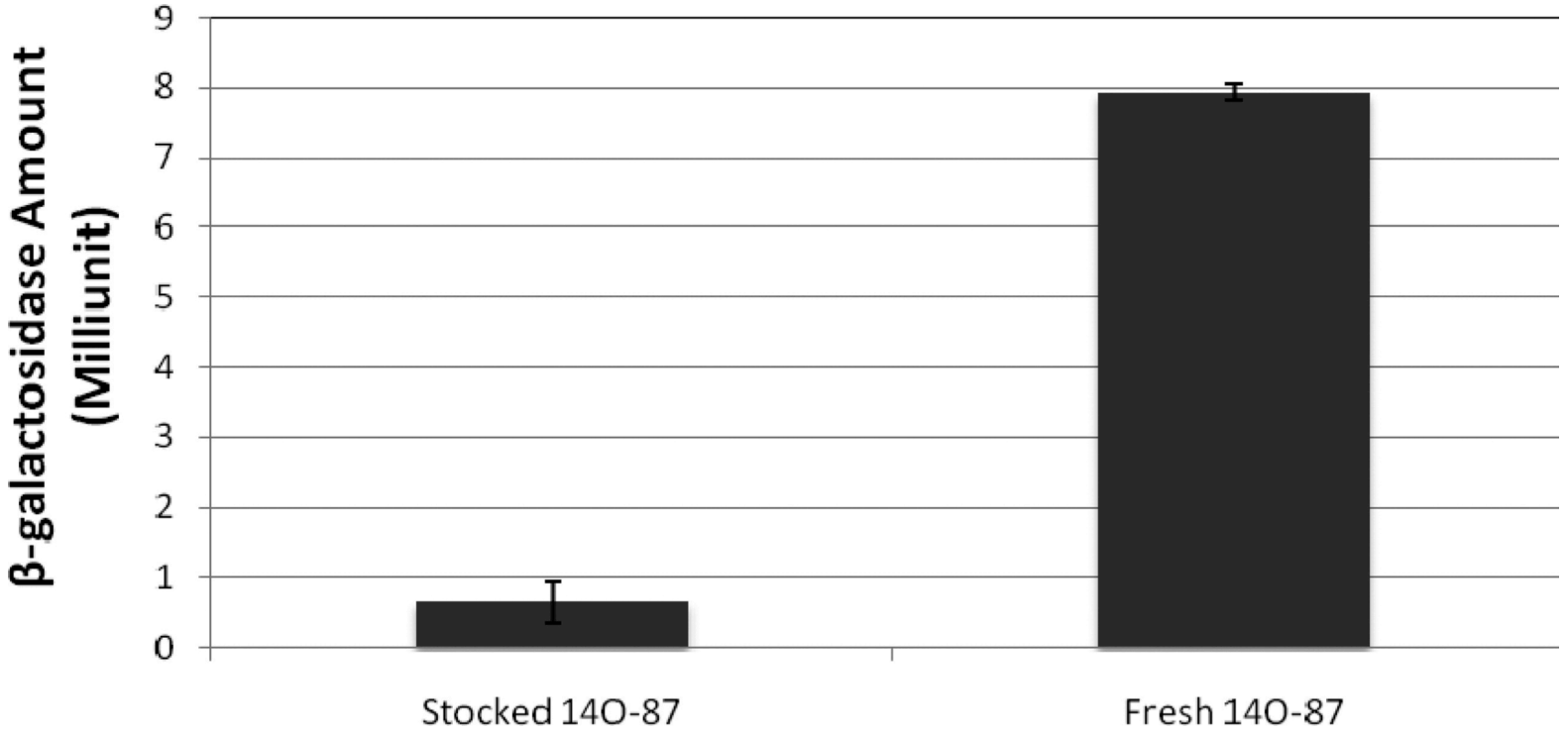


Figure 17. Different β-gal expression level delivered by fresh and stocked 14O-87/DNA complex. The delivery efficiency was converted from the β-gal activity assay. Lipidoid/DNA with a ratio o f 5:1 was used in the experiment.

The hydrolysis of acrylate (14O-87) derived lipidoids results in the loss of their lipid-like structure and thus the inability to assemble with DNA to form nanoparticles for delivery. It has been observed that the rapid degradation of amine-containing polyesters is attributed to the intramolecular nucleophilc assistance by the pendant amine groups[45-47]. In the case of 14O-87, we speculate that the rapid hydrolysis attributed to the nucleophilic attack of the free hydroxyl group of the molecule itself or nearby molecules.

2.4 Conclusions

We report the evaluation of a library of lipidoids for *in vitro* DNA delivery through a facile combinatorial approach. The mild reactions between amines and acrylamides, acrylates, or epoxides enabled the construction of a library to screen for efficient gene delivery carriers. The initial screening for DNA delivery indicated that lipidoids formed through amine and acrylamide addition reactions generated higher delivery efficiencies than acrylate- and epoxide-derived counterparts. The rapid hydrolysis of lipidoids with ester linkages decreased efficiency in DNA delivery. The structure-activity relationships demonstrated provide insight for designing new lipidoids for gene delivery. These lipidoids are easy to synthesize and do not require a co-lipid for efficient DNA delivery. We believe these systems could offer an inexpensive and effective alternative to other commonly used commercial gene delivery agents.

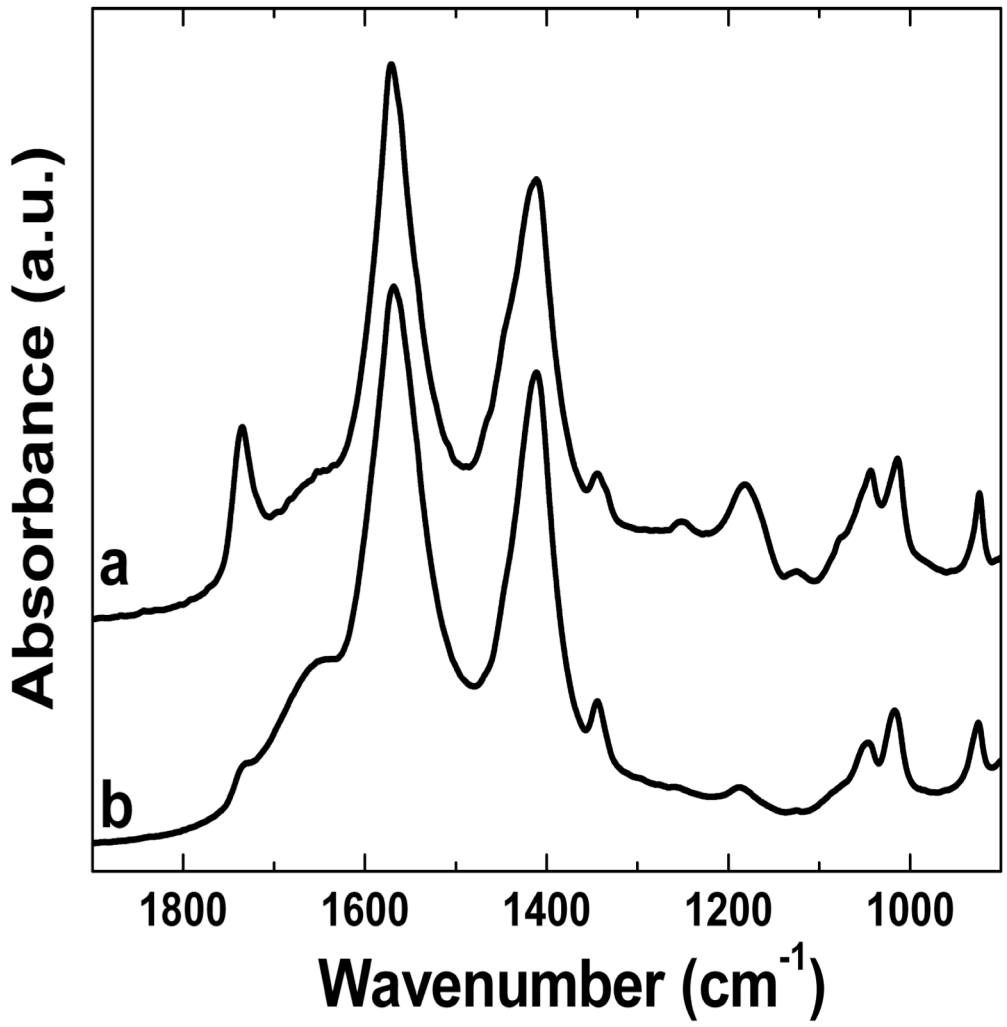


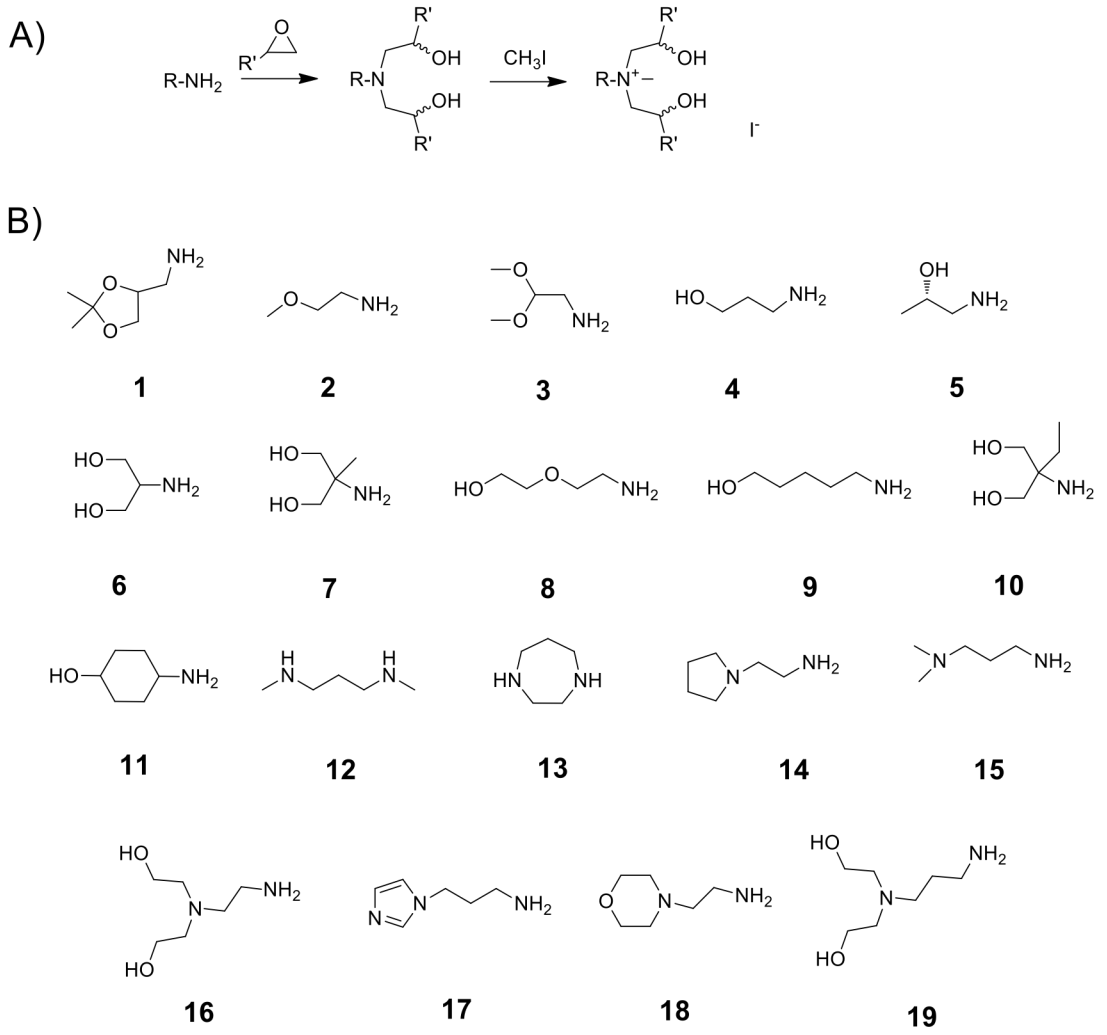
Figure 18. Normalized FT-IR spectra of fresh 14O-87(a) and stocked (b) solution. 14O-87 was dissolved in sodium acetate buffer solution (50 mM, pH = 5.5).

CHAPTER 3 A COMBINATORIAL LIBRARY OF QUATERNIZED LIPIDOID FOR IN VITRO DNA DELIVERY

3.1 Background

Recently, a combinatorial library of lipid-like materials termed "lipidoids" was developed and screened for their abilities to facilitate DNA and siRNA delivery[27, 34, 35, 48-50]. A variety of available amines, acrylamides, acrylates, or epoxides as reactants have allowed the chemical synthesis of structurally diverse lipidoids and the evaluation of structure-activity relationship of lipoidoids in gene delivery. The easy, economic combinatorial synthesis strategy and high delivery efficiency are making lipidoids a novel generation of non-viral delivery vectors.

In our previous work, we have found that lipidoids incorporating a secondary amine and two tails of 14 carbons are superior DNA transfection agents, in which a co-lipid is not necessary for efficient DNA delivery[48]. However, lipidoids incorporating a quaternary ammonium group, which have similar structures to classic cationic lipids, are not good gene transfection agents. The weak transfection abilities of quaternized lipidoids may arise from their strong electrostatic binding with genes that prohibit efficient intracellular gene release. To better probe the structure-activity relationship of lipoidoids in gene delivery and develop new materials for gene therapies, we here developed a combinatorial library of quaternized lipidoids through reacting a variety of amines with 1, 2-epoxyhexadecane, followed by the quaternization of ternary amines (Scheme 2). The quaternized lipidoids were then formulated with a neutral helper lipid, (DOPE) 1, 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (Figure 19), to form a library of liposomes



Scheme 2. (A) Combinatorial synthesis of quaternized lipidoids for DNA delivery; (B) Amine molecules used for the library synthesis; (C) Alkyl-epoxide used for the library synthesis Lipidoids are named as follows: (Quaternized Lipidoids (QD)) (carbon numbers of tail)-(amine number)

and screened their abilities to facilitate gene delivery. We found that the formulation of DOPE can enhance the DNA transfection abilities of quaternized lipidoids, some of them even transfected HeLa cells in higher efficiency than the commercial transfection agent, Lipofectamine 2000. The enhanced DNA transfection of quaternized lipidoid/DOPE liposomes may be benefiting from the weak lipidoid-DNA binding due to the decrease of positive charge density of lipidoids and the hexagonal-phase forming propensity of liposomes. We further optimized and investigated the liposome composition and DNA doses for gene transfection as well as the structure-activity relationships of the quaternized lipidoids library in DNA delivery.

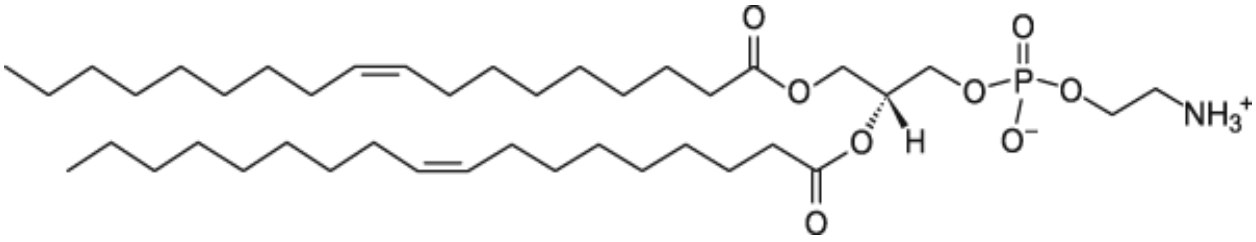


Figure 19. **Structure of helper lipid DOPE**

3.2. Materials and Methods

3.2.1 Materials

All chemicals for lipidoid synthesis were purchased from Sigma-Aldrich, TCI, or Alfa-Aesar and used directly as received. pCMV *β*-gal DNA was provided by Elim Biopharmaceuticals, Inc (Hayward, CA). Triton and *o*-nitrophenyl-*β*-D-galactoside used for *β*-gal activity assay were purchased from Sigma-Aldrich. Picogreen assay kit was

obtained from Molecular Probes (Invitrogen, Carlsbad, CA).

3.2.2 Synthesis of lipidoids

The synthesis lipidoids was performed and characterized as previously described[35]. Generally, to a 5-mL Teflon-lined glass screw-top vial was added amine and 1, 2-epoxyhexadecane in a molar ratio of 1:2.4, followed by a further 3 days of stirring at 90°C. After cooling, the reaction mixtures were purified unless otherwise specified. The purified lipidoids were dissolved in THF and reacted with excessive amount of methyl iodide overnight at room temperature in the dark. The obtained precipitations were filtered, washed with diethyl ether and dried in vacuum for further using. Representative lipidoid was characterized by thin layer chromatography, IR, ^{1}H NMR, and mass spectroscopy.

3.2.3 Liposome formulation

Quaternized lipidoids and DOPE liposomes were formulated as described by Huang et al. with slight modification[51]. Briefly, as shown in Figure 17, the desired amounts of lipidoids and DOPE were dissolved with chloroform in a glass vial, the organic solvent was then evaporated under reduced pressure to form a thin film on the bottom of the vial. A sodium acetate buffer solution (25 mM, pH = 5.5) was added to the vial, the suspension was sonicated for 15 minutes until a clear and uniform suspension obtained. The suspension was kept in the fridge overnight to allow for complete hydration prior to use.

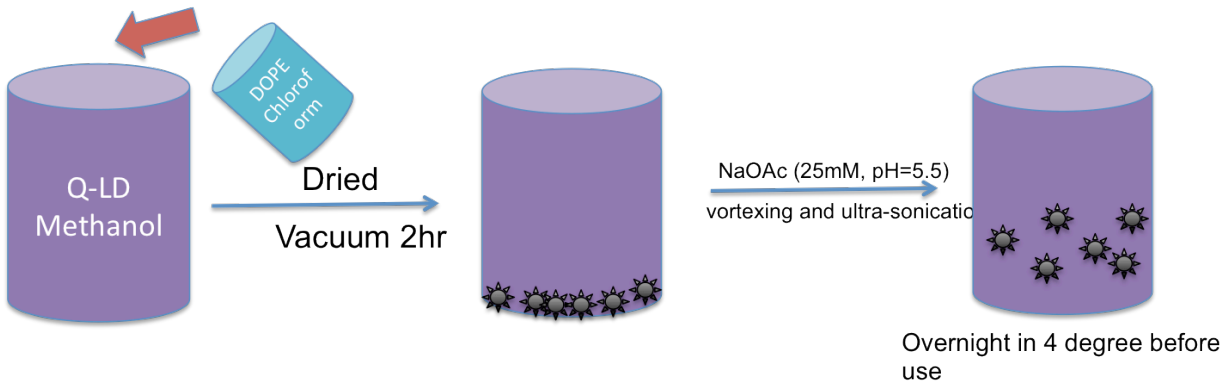


Figure 20. Illustration of liposome Formulation

3.2.4 Cell culture

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. HeLa cells were obtained from ATCC (Manassas, VA) and cultured in high glucose DMEM supplemented with 10% fetal bovine serum and 100 units/mL of penicillin/streptomycin at 37°C in the presence of 5% CO_2. Cells were seeded into 96-well plates at a density of 10,000 cells per well one day prior to transfection experiment.

3.2.5 In Vitro DNA Transfection

To facilitate the rapid throughput screening of lipidoids for DNA delivery, quaternized lipidoid/DOPE liposomes were mixed with DNA in sodium acetate buffer solutions (25 mM, pH = 5.5) at a fixed P/N ratio of 2:1(lipidoid:DNA), followed by 15 minutes of incubations at room temperature to allow for complete lipoplexes formation. For transfection experiments in 96-well plates, cells were transfected with lipoplexes of 200 ng of DNA (per well) at fixed liposome/DNA ratios. After a further 48 h of incubation at 37°C, cells were lysed and assayed for β-gal activity. Control experiments were performed by using Lipofectamine 2000 as per the manufacturer's instructions. All

transfection experiments were performed in quadruplicate.

3.2.6 *β*-gal activity assay

The *β*-gal activity was assayed according to previous method with slight modification[40]. Briefly, after the cell transfection experiment over 48 hours, all growth medium was removed by vacuum aspiration, wash the cells with PBS for two times and lyse the cells with 0.5% triton (50 mL/well in PBS), then 50 mL of *o*-nitrophenyl-*β*-D-galactoside (ONPG) solution (4 mg/mL in z-buffer) was added, followed by a 15 min. of at 37°C to allow the enzymatic hydrolysis of ONPG. At the end of incubation, the concentration of *β*-gal was determined by measuring the absorbance at 409 nm.

3.2.7 DNA encapsulation assay

The DNA encapsulation efficiency of liposomes was determined by PicoGreen assay as previously described[41]. Briefly, 50 mL/well of liposome solution (1 mg/mL in NaOAc buffer solution, 25 mM, pH = 5.5) and 50 mL/well of DNA (60 mg/mL in NaOAc buffer solution, 25 mM, pH = 5.5) were mixed in a 96-well plate, followed by a further 15 min. of incubation to allow for lipoplexes formation. Then, 100 mL/well of PicoGreen working solution (diluted 190 times from the Picogreen assay kit with NaOAc buffer solution, 25 mM, pH = 5.5) was added. After a 5 min. of incubation, 30 mL of lipoplex/PicoGreen solution was diluted by 200 mL DMEM in a black 96-well polystyrene plate, the fluorescence intensity of the solution was monitored at 535 nm by excitation at 485 nm on a SpectraMax® M2 Multi-Mode Microplate Reader (Molecular Devices, Inc. California, USA), as shown in Figure 21. The relative fluorescence (*RF*) was calculated by the following formula:

$$RF = (F_1\text{-}F_0)/(F_2\text{-}F_0)$$

Where: F_1 and F_2 are the fluorescence intensities of the DNA-PicoGreen complexes with and without liposomes, F_0 is the fluorescence intensity of PicoGreen solution. The DNA encapsulation ratio was calculated as (1- RF) × 100%.

Figure 21. Photo of SpectraMax® M2 Multi-Mode Microplate Reader

3.2.8 Transmission Electron Microscopy (TEM)

Lipidoid/DNA complexes were prepared as same as *in vitro* experiments. Droplets of the sample (5 mL) were applied to hydrophilized carbon-covered copper grids (300 meshes) for 30 min. The sample was subsequently rinsed with contrasting material (1% uranyl

acetate at pH 4.5). The remaining stain solution was removed with a filter paper and air-dried. TEM microstructure was determined using a Tecnai FEG TEM (FEI Tecnai 12 Spirit Bio-twin, FEI Company, Hillsboro, OR) operating at 80 kV.

3.2.9 *In vitro* cytotoxicity assay

The *in vitro* cytotoxicity of liposome/DNA was evaluated by MTT assay. Briefly, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was dissolved in phenol red free DMEM at a concentration of 0.5 mg/mL. Cells transfected with various lipoplexes were washed with warm DMEM, added with MTT solution (125 μL per well in a 96 well plate), followed by a 3h of incubation at 37°C. At the end of the incubation, the DMEM solution was removed and added with 125 μL DMSO (per well), the solutions were pipetted up and down several times to ensure the converted dye was completely dissolved. Absorbance of solutions was measured at 570nm with background subtraction at 650nm.

3.3. Results and Discussion

3.3.1 Preliminary screening of quaternized lipidoid/ DOPE liposomes for DNA delivery

Figure 22 shows the preliminary screening results by delivering *β*-gal encoding DNA into HeLa cells with liposomes comprised different quaternized lipidoids and DOPE. To better for the library screening, quaternized lipidoid and DOPE were formulated at a fixed molar ratio of 2:1, the lipidoid and DNA were mixed at a fixed

P/N ratio of 5:1. The β-gal activity assay indicated that quaternized lipidoids themselves can not facilitate efficient DNA delivery, however, after formulating with DOPE, the β-gal expression level was enhanced significantly except the lipidoids of QD16-1, QD16-7, and QD16-18. Three lipidoids QD16-15, QD16-17, and QD16-19 formulated liposomes can transfect the HeLa cells in comparable efficiency to the commercial agent, Lipofectamine 2000.

3.3.2 DOPE promotes the transfection efficiency of lipidoids

The preliminary screening results indicated that the formulation of DOPE can enhance the transfection abilities of quaternized lipidoids. We next investigated the effect of lipidoid/DOPE ratios on the transfection efficiency. QD16-15, QD16-17, and QD16-19were selected for detail studies due to their high transfection performance in the library screening. Liposomes formulated from quaternized lipidoids and DOPE in molar ratios varied from 1:8 to 10:1 (Lipidoid:DOPE) were tested for their abilities to facilitate DNA delivery. As shown in Figure 23, the increase of DOPE ratio in the liposomes can enhance the transfection efficiency until a highest β-gal activity at a molar ratio of 1:2, a further increase of DOPE ratio weaken the transfection abilities of liposome significantly. The enhanced transfection abilities of quaternized lipidoid formulated with DOPE compared to lipidoid itself may result from the hexagonal-phase-forming propensity of liposomes when interact with cell membranes[52-55]. Meanwhile, the formulation of DOPE may also neutralize the positive charge densities of lipidoids and weaken the lipidoid and DNA binding due to the competition interactions of DOPE and DNA, which may further facilitate intracellular gene release[55, 56].

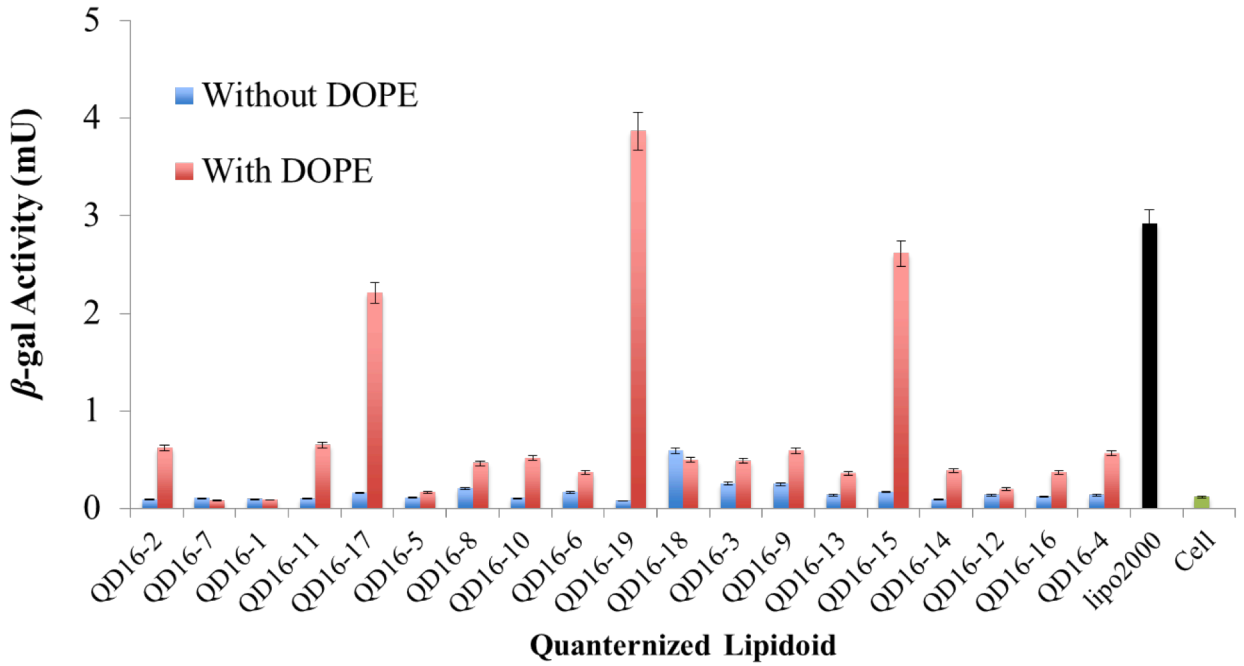


Figure 21. Initial in vitro screening of quaternized lipidoids formulated with (quaternized lipidoids/DOPE molar ratio was 1:2) and without DOPE for DNA delivery. Lipidoids were screened by delivering β-gal encoding plasmid DNA into HeLa cells. Relative β-gal expression level was determined by assaying the enzyme activity according to reported methods, for details see experimental section.

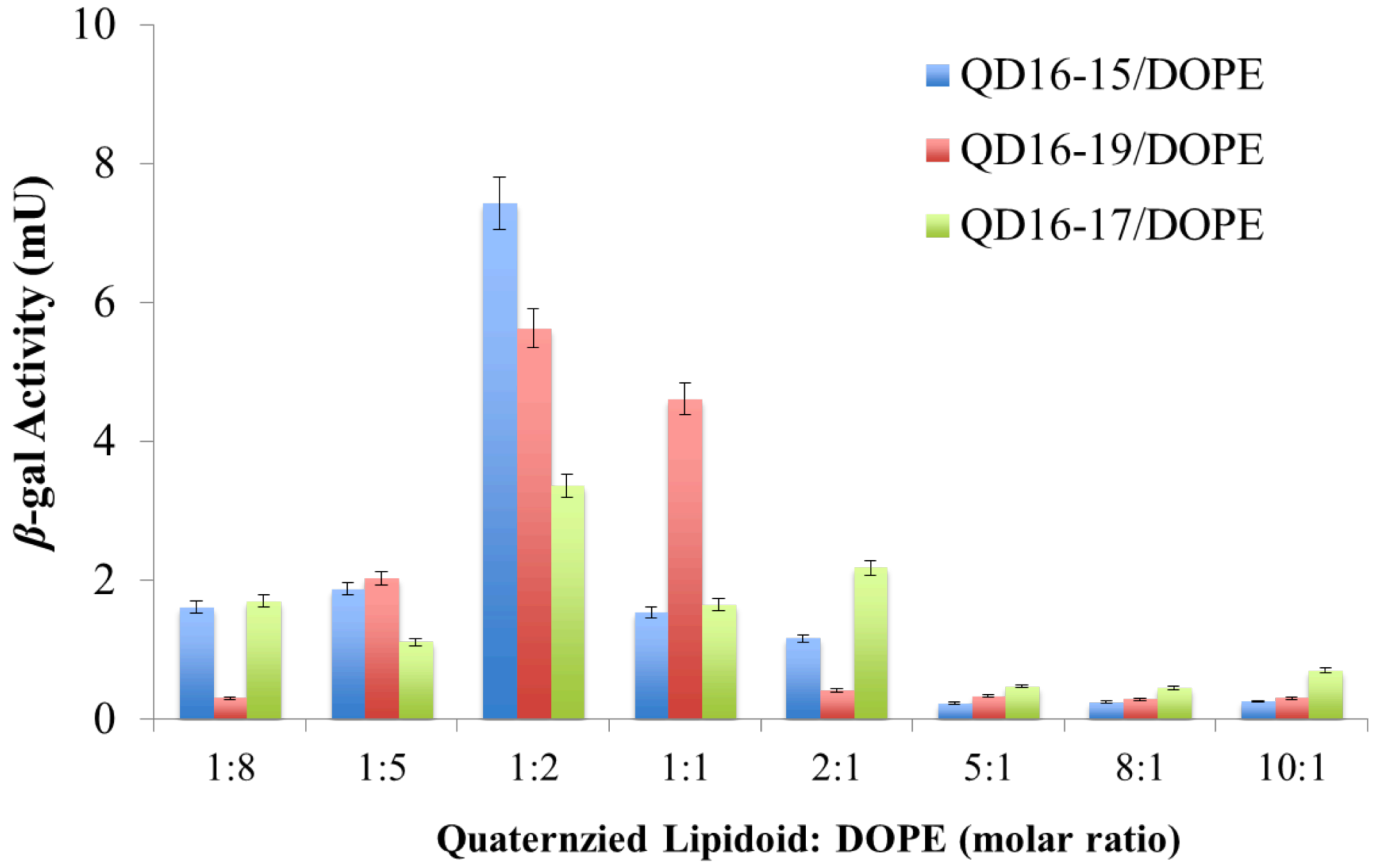


Figure 23 .The effect of quaternized lipidoids/DOPE molar ratios on DNA delivery efficiency (Liposome/DNA ratio was fixed at the P/N of 2:1). The efficiency was monitored as β-gal expression level.

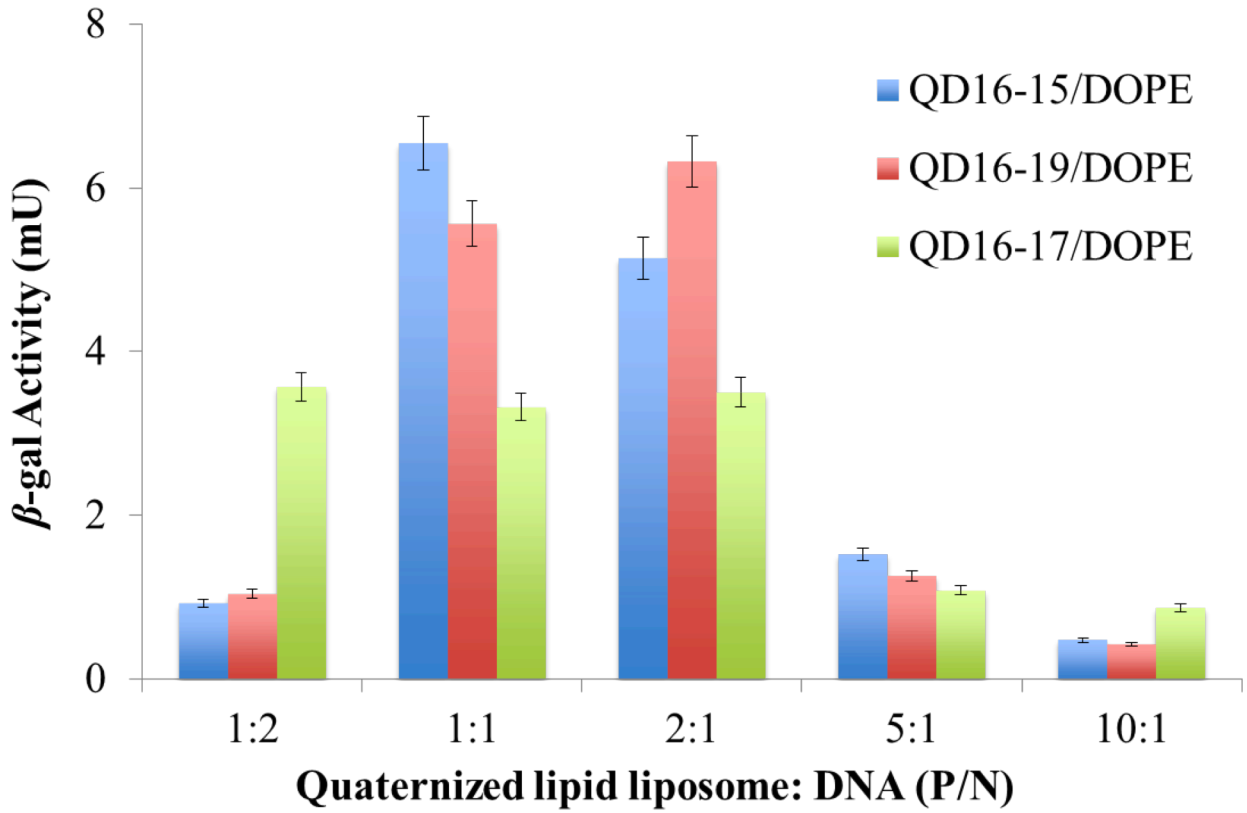


Figure 24. The effect of quaternized lipidoids: DNA ratios on gene transfection efficiency (Quaternized lipidoids and DOPE were formulated at a molar ratio of 1:2). The transfection efficiency was monitored as β-gal expression level.

By fixing the quaternized lipidoid and DOPE at a 1:2 molar ratio, we further investigated the effect of lipidoid/DNA ratios on the transfection abilities of the liposomes. As indicated in Figure 24, by increasing the lipidoid/DNA ratio(weight/weight) from 1:2 to 2:1, QD16-19/DOPE increased the transfection efficiency until a highest β-gal activity at the lipidoid/DNA ratio of 2:1, while QD16-15 and QD16-17 liposomes reached the highest transfection efficiency at a lipidoid/DNA ratio of 1:1. The higher transfection efficiency of at lipidoid/DNA ratios above may be ascribed to the higher DNA encapsulation and cellular uptake, which can be confirmed by a Picogreen assay. As shown in Figure 25, liposomes with a low lipidoid/DNA ratio (<1:2) displayed DNA encapsulation efficiency lower than 20%. An increasing of lipidoid/DNA ratio can enhance the DNA encapsulation efficiency until the highest at a 5:1, in which QD16-15 and QD16-19 liposomes can encapsulate DNA at nearly 100%, a 70% encapsulation rate was observed for QD16-17. The higher DNA encapsulation enabled the higher cellular uptake and gene transfection.

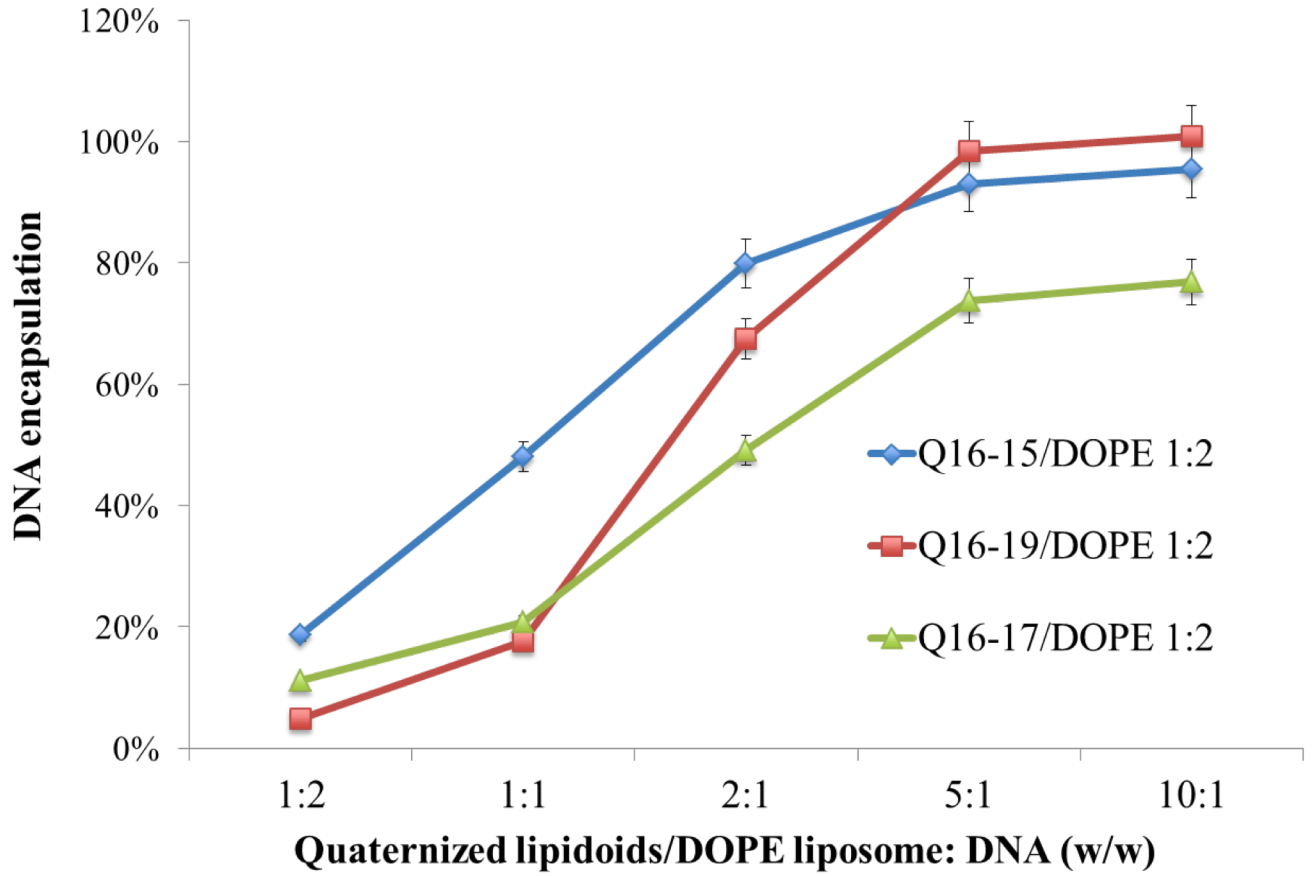


Figure 25. The effect of quaternized lipidoids: DNA ratios on the DNA binding efficiency.(Quaternized lipidoids and DOPE were formulated at a molar ratio of 1:2). The DNA encapsulation efficiency was determined by Picogreen assay.

3.3.3 Morphology of lipidoid/DOPE-DNA lipoplexes

It has been reported that the transfection ability of a given lipid or liposome is largely dependent on the lipoplex structures and physicochemical properties[44]. We here used negative-staining transmission electron microscopy (TEM) to characterize the size and morphology of the DNA and lipidoid complexes formulate with and without DOPE, as a way to illustrate the DOPE promoted DNA delivery facilitated by quaternized lipidoids. As shown in Figure 26A, the QD16-15/DOPE liposome was able to self-assemble with DNA to form condensed multilamellar structure with size around 100 nm, in which DNA was intercalated between the lipid bilayers. However, QD16-15 itself can only form large aggregation clusters (2~3 micrometers) with DNA binding (Figure 26C), though multilamellar structure nanocomplexes (Figure 26D) could also be observed. The different lipoplex structures induced cellular uptake of lipoplex and gene transfection in different efficiency, it has been recognized that usually cause a higher cellular uptake than the particles in micrometer size.

3.3.4 Effect of lipidoids tail length on DNA transfection efficiency

To further investigate the structure-activity relationship of lipidoids in facilitating DNA delivery, head amines of **15**, **17** and **19**, which generate lipidoids with high transfection efficiency in the library, were selected and reacted with epoxides of different tail lengths. The purified lipidoids were quaternized, formulated with DOPE at the optimized ratio indicated above and tested for DNA delivery. As shown in Figure 27, all lipidoids incorporating two tails of 12- or 14-carbon transfected HeLa cells in low efficiency, amine **15** and **19** derived lipidoids with 16-carbon tails, **17** with two tails of 18-carbon

lipidoid transfected HeLa cells in the highest efficiency. The transfection difference may due to the varied DNA-lipidoid interactions and DNA encapsulation efficiency arising from the tail rigidities of lipidoids. The interaction of DNA with the relatively rigid 12- and 14- carbon tail lipidoids, DNA was inappropriately condensed and formed structurally deformed lipoplexes[57], which hampered plasmid translocation across endosomal and nuclear membranes. The DNA encapsulation efficiency of liposomes comprised lipidoids of different tail lengths was determined by PicoGreen assay to better illustrate the effect of tail length of lipidoid on the transfection efficiency. As shown in Figure 28, the liposomes derived from lipidoid with 12 or 14 carbon tails encapsulated DNA in low efficiency except the QD14-19, while lipidoids with 16 or 18-carbon tails increased the DNA encapsulation efficiency greatly. The increased DNA encapsulation efficiency of lipidoids with long tail length correlated well with the high transfection efficiency.

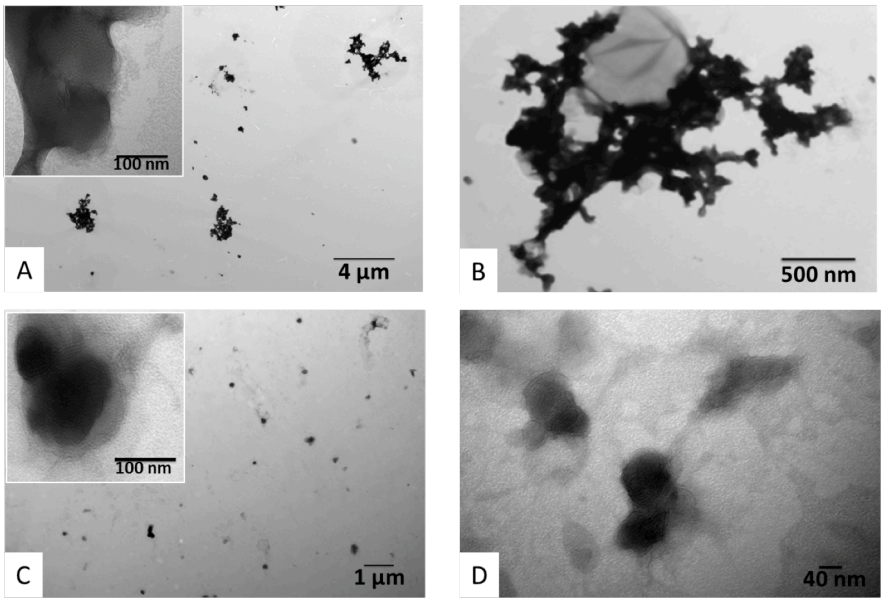


Figure 26. Negative staining TEM images of lipoplexes of QD16-15/DOPE and DNA (A and B); QD16-15/DNA complex (C and D).

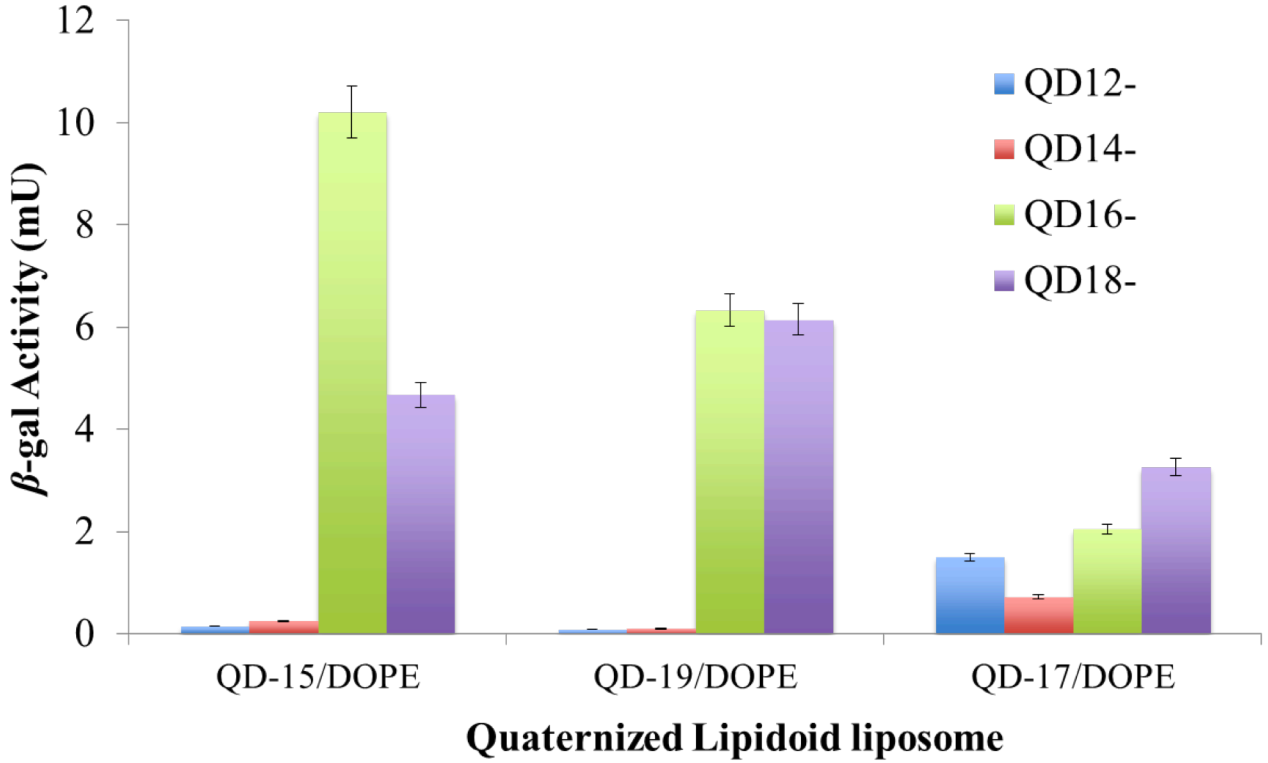


Figure 27. The effect of lipidoids tail length on the DNA transfection efficiency. Quaternized lipidoids were formulated with DOPE at molar ratios of 1:2; Lipoplexes were prepared by a lipidoid:DNA ratio of 2:1(P/N). The efficiency was expressed as β-gal activity.

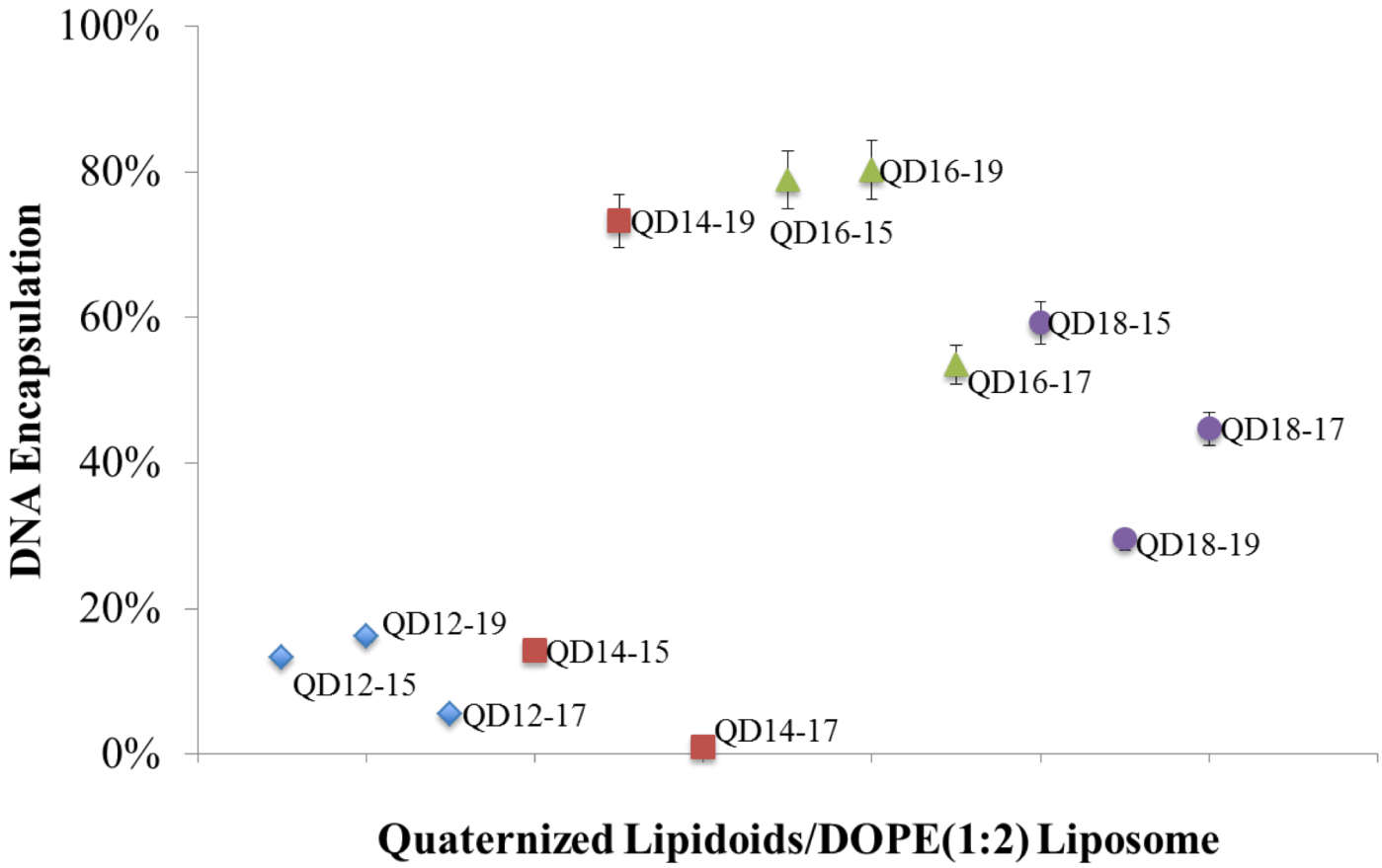


Figure 28. The effect of lipidoids tail length on the DNA encapsulation efficiency. Quaternized lipidoids were formulated with DOPE at molar ratios of 1:2; Lipoplexes were prepared by a lipidoid:DNA ratio of 2:1(P/N). The DNA encapsulation efficiency was determined by Picogreen assay.

3.3.5 Effect of DNA dose on transfection efficiency

By fixing the lipidoid/DNA at ratios of 2:1 and the liposome formulations, the increase of DNA dose led to the enhancement of β-gal expression level. As shown in Figure 29, with the increasing of DNA dose from 10 ng to 100 ng per well, the protein expression level increased gradually. Higher DNA dose have no effect on the delivery efficiency even decrease the β-gal activity due to the increased cytotoxicity of large amount of lipidoids(Figure 30).

3.4 Conclusions

In this project, we described the combinatorial synthesis of a quaternized lipidoid library and formulation with DOPE to form liposomes to facilitate intracellular DNA delivery. The formulated quaternized lipidoids can enhance their transfection abilities greatly, partly because of the decreased liposome-DNA binding that facilitate intracellular gene release and the hexagonal-phase-forming propensity of. We further investigated the effect of liposomes composition, the lipidoid/DNA ratio, and the DNA dose on the gene transfection efficiency. The structure-activity studies indicated that quaternized lipidoids with two tails of 16 to 18 carbons are superior to the ones with short tails. The design rationales demonstrated in the paper will provide new principles to develop new materials for gene delivery and therapy with high efficiency.

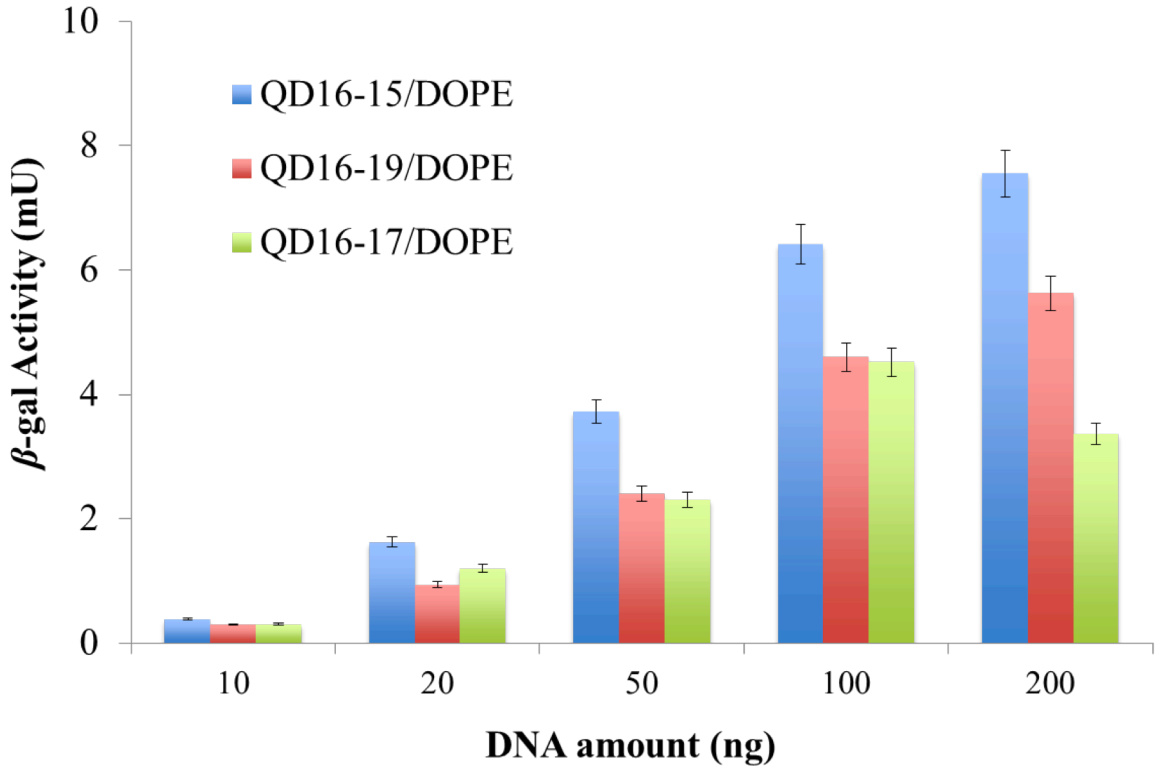


Figure 29. The relationship of DNA amount and *β*-gal activity. Quaternized lipidoids were formulated with DOPE at molar ratios of 1:2; Lipoplexes were prepared by a lipidoid:DNA ratio of 2:1(P/N).

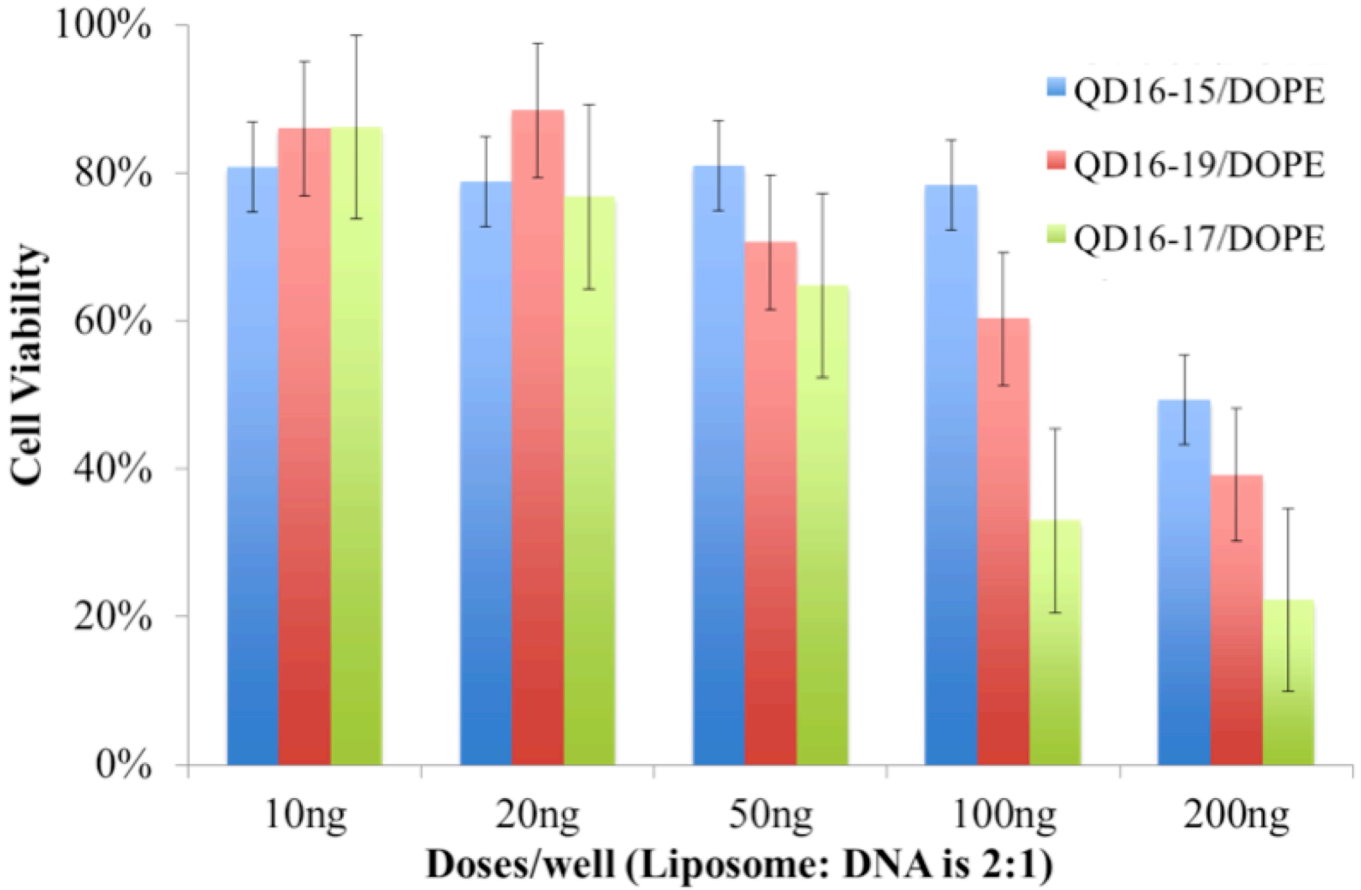


Figure 30. The relationship of DNA amount and cell viablility. Quaternized lipidoids were formulated with DOPE at molar ratios of 1:2; Lipoplexes were prepared by a lipidoid:DNA ratio of 2:1(P/N).

SUMMARY AND FUTURE WORK

A library of lipidoids for in vitro DNA delivery through a facile combinatorial approach was investigaed. The mild reactions between amines and acrylamides, acrylates, or epoxides enabled the construction of a library to screen for efficient gene delivery carriers. The initial screening for DNA delivery indicated that lipidoids formed through amine and acrylamide addition reactions generated higher delivery efficiencies than acrylate- and epoxide-derived counterparts. The rapid hydrolysis of lipidoids with ester linkages decreased efficiency in DNA delivery. The structure-activity relationships demonstrated provide insight for designing new lipidoids for gene delivery. These lipidoids are easy to synthesize and do not require a co-lipid for efficient DNA delivery. We believe these systems could offer an inexpensive and effective alternative to other commonly used commercial gene delivery agents.

In addition, a quaternized lipidoid library and formulation with DOPE to form liposomes to facilitate intracellular DNA delivery was evaluated. DOPE can enhance the DNA transfection abilities of quaternized lipidoids, some of them even transfected HeLa cells in higher efficiency than the commercial transfection agent, Lipofectamine 2000. The enhanced DNA transfection of quaternized lipidoid/DOPE liposomes may be benefiting from the weak lipidoid-DNA binding due to the decrease of positive charge density of lipidoids and the hexagonal-phase forming propensity of liposomes.

Future work may focus on the following directions. Firstly, we will continue to examine the structure-activity relationship of lipidoids for gene delivery, which may benefit us for the rational design of novel materials for more efficient delivery. Secondly,

although lipidoids have been used for both in vitro and in vivo gene delivery, it is still significantly necessary to investigate how to decrease the toxicity of lipidoids.

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