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Charge-Reversal Lipids, Peptide-Based Lipids, and Nucleoside-Based Lipids for Gene Delivery

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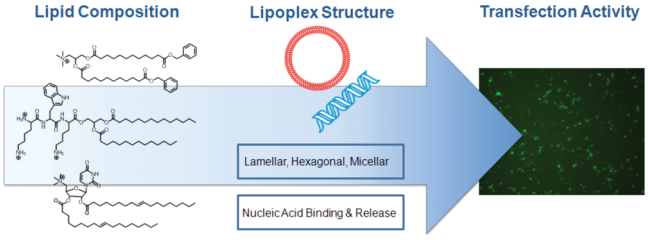

Twenty years after gene therapy was introduced in the clinic, advances in the technique continue to garner headlines as successes pique the interest of clinicians, researchers, and the public. Gene therapy's appeal stems from its potential to revolutionize modern medical therapeutics by offering solutions to myriad diseases through treatments tailored to a specific individual's genetic code. Both viral and non-viral vectors have been used in the clinic, but the low transfection efficiencies when non-viral vectors are used have lead to an increased focus on engineering new gene delivery vectors. To address the challenges facing non-viral or synthetic vectors, specifically lipid-based carriers, we have focused on three main themes throughout our research: (1) The release of the nucleic acid from the carrier will increase gene transfection. (2) The use of biologically inspired designs, such as DNA binding proteins, to create lipids with peptide-based headgroups will improve delivery. (3) Mimicking the natural binding patterns observed within DNA, by using lipids having a nucleoside headgroup, will produce unique supramolecular assembles with high transfection efficiencies.

The results presented in this Account demonstrate that engineering the chemical components of the lipid vectors to enhance nucleic acid binding and release kinetics can improve the cellular uptake and transfection efficacy of nucleic acids. Specifically, our research has shown that the incorporation of a charge-reversal moiety to initiate a shift of the lipid from positive to negative net charge improves transfection. In addition, by varying the composition of the spacer (rigid, flexible, short, long, or aromatic) between the cationic headgroup and the hydrophobic chains, we can tailor lipids to interact with different nucleic acids (DNA, RNA, siRNA) and accordingly affect delivery, uptake outcomes, and transfection efficiency. The introduction of a peptide headgroup into the lipid provides a mechanism to affect the binding of the lipid to the nucleic acid, to influence the supramolecular lipoplex structure, and to enhance gene transfection activity. Lastly, we discuss the *in vitro* successes that we have had when using lipids possessing a nucleoside headgroup to create unique self-assembled structures and to deliver DNA to cells. In this Account, we state our hypotheses and design elements as well as describe the techniques that we have used in our research to provide readers with the tools to characterize and engineer new vectors.

1. Introduction

Many hereditary and nonhereditary diseases caused by chromosomal deficiencies and anomalies (e.g., mutation, aberrant expression) are currently untreatable using conventional medicine. Consequently, a number of new ideas and techniques are being explored to meet this need,

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including gene therapy. Gene therapy, which acts by adding, replacing, repairing, or removing genes, is an effective therapeutic option that has seen multiple clinical successes.[1] It has been over 20 years since the inaugural gene therapy clinical trials. The successful treatment of adenosine deaminase (ADA) deficiency using retroviral-mediated gene transfer demonstrated the potential therapeutic benefits of this new technique.[2,3] Since then, patients have been treated for a wide variety of diseases ranging from neurological diseases to cancers.[4] Consequently, gene therapy is a treatment modality that is being practiced globally. In 2003, a recombinant adenovirus–p53 gene therapy (Gendicine) for head and neck squamous cell carcinoma was approved for commercial production and for use in China.[5] This achievement provides further motivation for continued research and clinical studies to advance gene-based therapies for diseases such as AIDS, cystic fibrosis, venous ulcers, glaucoma, and cancers.

The delivery of nucleic acids (i.e., DNA, RNA, siRNA, and ODN) into cells is a key step in gene therapy. Traditionally, DNA delivery has been classified into two major categories based on the delivery vehicle: viral[1,6,7] and nonviral[8–16] vectors. Viral vectors are the most effective means to deliver DNA, due to the highly evolved and specialized components present within viruses, and are currently the dominant approach used in clinical trials worldwide. Overall, viral mediated gene therapy is a very active and important area for research, development, and commercialization.

Our interest is in cationic lipids as synthetic, nonviral vectors because (1) there is precedence for their *in vitro* and *in vivo* use to deliver nucleic acids, (2) they can complex small and large molecular weight nucleic acid samples, including those that have been chemically modified, (3) they are discrete, well-defined chemical entities that can be fully characterized, (4) the chemical structure can be altered to optimize vectors, and (5) the synthesis can be scaled for low-cost, quality-controlled manufacturing. Yet, cationic amphiphiles exhibit low transfection efficiencies compared with viruses. In our opinion, this low activity reflects an unoptimized lipid/nucleic acid complex, in terms of structure, function, or both, which leads to inefficiencies in the transfection pathway. This pathway includes DNA–synthetic vector complexation, endocytosis, endosomal escape, nuclear entry, and expression. Therefore, a number of research groups, including ours, have focused on developing new vectors to address these limitations.

To overcome the transfection barriers and obtain higher gene delivery efficiencies, we are testing the following hypotheses that address the above challenges.

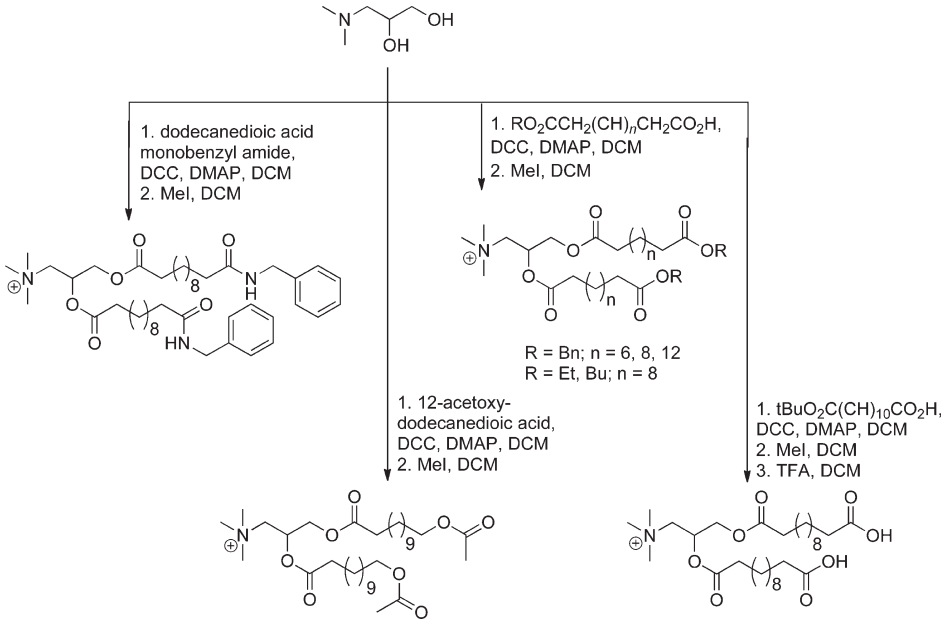
Hypothesis 1. The performance of cationic lipid vectors can be improved by incorporating a charge-reversal or charge-switching feature in the lipid so that the cationic vector initially binds DNA and then releases DNA due to a change in vector charge to anionic. This hypothesis addresses the idea that once a vector complexes to nucleic acids it has minimal favorable enthalpic or entropic energies to release it within the cell.

Hypothesis 2. A cationic vector headgroup that binds DNA through interactions seen in native systems, such as with DNA binding proteins, will show less cytotoxicity and perform better than one composed of a conventional cationic group (e.g., choline). This hypothesis addresses the idea that both electrostatic and π-stacking interactions, as well as their spacing in a vector, will influence DNA complexation and transfection activity.

Hypothesis 3. Nucleoside-based lipids, which combine the molecular recognition principles seen in a DNA duplex with the self-assembly characteristics of lipids, provide a unique approach to prepare supramolecular systems with tunable physicochemical properties for gene delivery. This addresses the idea that a cationic vector that binds DNA through Watson–Crick-type or Hoogsteen interactions, as seen in DNA itself, will perform better than one composed of a conventional cationic group (e.g., choline).

Consequently, the long-term goals of our research effort are fourfold: (1) to synthesize new lipids that possess responsive characteristics or can bind to nucleic acid via multiple different noncovalent interactions, (2) to elucidate the mechanism of cellular uptake and nucleic acid delivery, (3) to rationally design an efficient transfection vehicle based on structure–property–activity relationships, and (4) to evaluate an optimized nucleic acid delivery vector in both *in vitro* and *in vivo* experiments. We have made progress on several of these goals, and this Account highlights our findings. We begin with a general description of the synthetic routes to these lipids, followed by a discussion of the physicochemical properties and transfection capability of the three different

SCHEME 1. Synthetic Route to the Charge-Reversal Lipids Possessing a Simple Quaternary Amine and Their Counterparts



classes of lipid: charge-reversal, peptide-based, and nucleoside-based lipids.

2. Synthetic Routes to the Lipids

The lipids under investigation possess three components, each with specific functions: the headgroup, the backbone, and the hydrocarbon chains. The headgroup is responsible for the affinity to the nucleic acid through electrostatic, H-bonding, or π-stacking interactions. The backbone provides a structural unit to orient the headgroup, as well as to connect the chains. A bilayer is created by the chains to stabilize the resulting lipid–nucleic acid supramolecular assembly. Our preferred synthetic route to these lipids involves a convergent approach where the backbone and chains are prepared first and then coupled to the headgroup, as shown in Schemes 1–5.

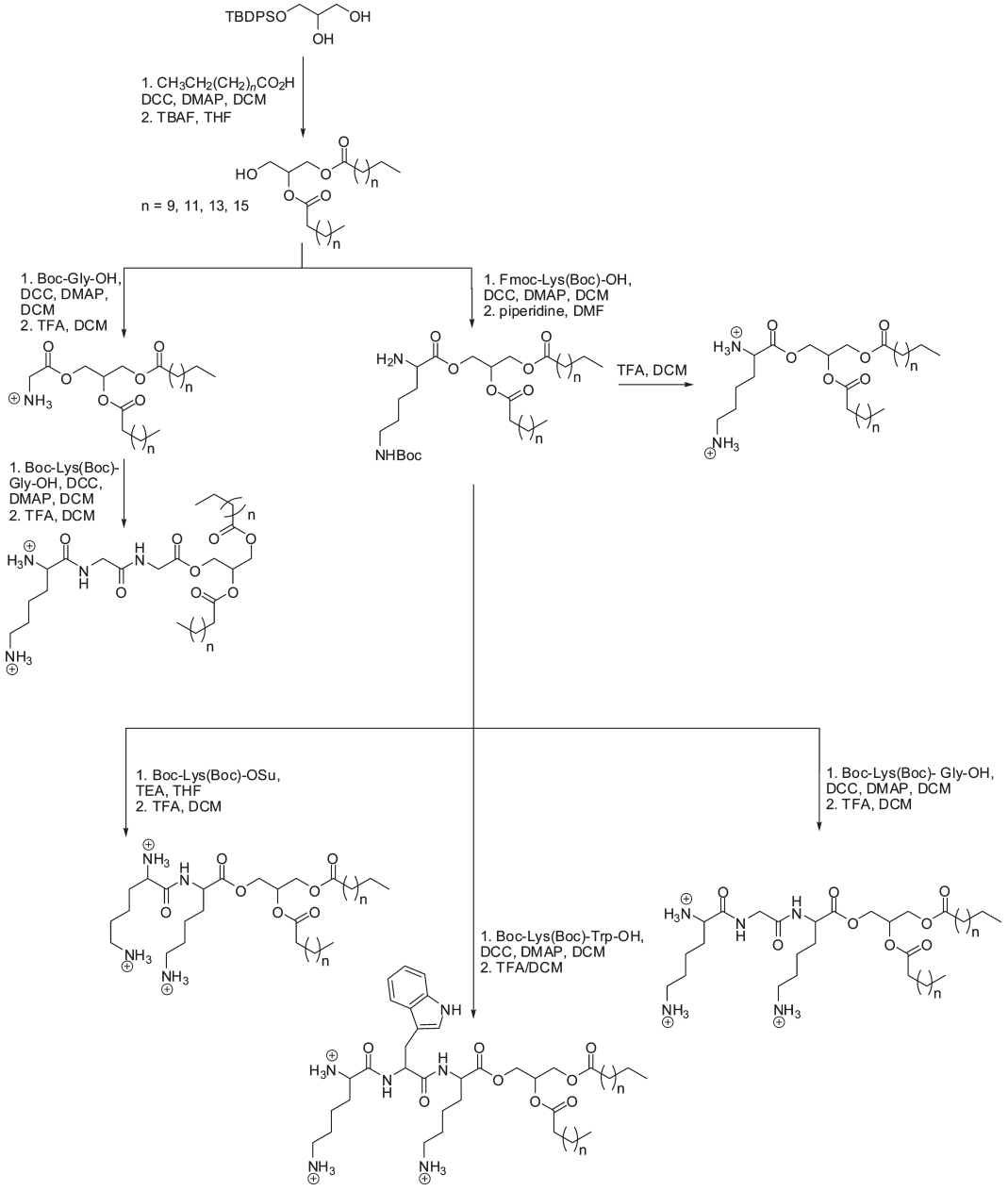
The structurally simplest charge-reversal lipids are prepared by coupling the free hydroxyl groups of 3-(dimethylamino)-propane-1,2-diol with the appropriate long-chain carboxylic acid derivatives (commonly monoesters of dodecanedioic acid) using DCC as a condensation agent (Scheme 1). Subsequent formation of the cationic lipids is achieved by reaction of the amine moiety with iodomethane forming the quaternary amine salts.[17–20]

To synthesize peptide-functionalized cationic lipids, monosilyl-protected glycerol is esterified at the secondary and the remaining primary hydroxyl positions using established carbodiimide chemistry (Scheme 2). Following silyl group deprotection, the remaining primary hydroxyl group is coupled to a glycine or lysine derivative. The amino acid-functionalized lipid is then further functionalized by sequential carbodiimide facilitated couplings with amino acid derivatives, such as those of glycine, lysine, or tryptophan. Acid deprotection of the amino acid protecting groups affords singly or multiply charged peptide-based cationic lipids.[21]

Similarly, the monosilyl-protected glycerol can be esterified using dodecanoic acid monobenzyl ester in a similar fashion (Scheme 3). Following the removal of the silyl group, the free hydroxyl can be converted to dioxaphospholane-oxide, which after reaction with trimethylamine allows for a facile formation of the zwitterionic phosphate charge-reversal lipid. Alternatively, the free hydroxyl group can be esterified by a number of "dimethylamino-*spacer*-carboxylic acid" derivatives, using carbodiimide chemistry. The prepared amine-functionalized molecules are subsequently reacted with iodomethane to form quaternary amine salts, affording cationic charge-reversal lipids with a range of headgroups.[20]

The cationic nucleoside-based lipids are prepared by first protecting the 2′ and 3′ hydroxyl groups of the ribose moiety with an acetonide protecting group (Scheme 4). The 5′-OH is subsequently functionalized as a tosylate, allowing for formation of a quaternary amine salt at the 5′ position of the ribose following a nucleophilic substitution reaction with trimethylamine. Removal of the 2′,3′-O-isopropylidene

SCHEME 2. Synthetic Route to the Peptide-Based Lipids

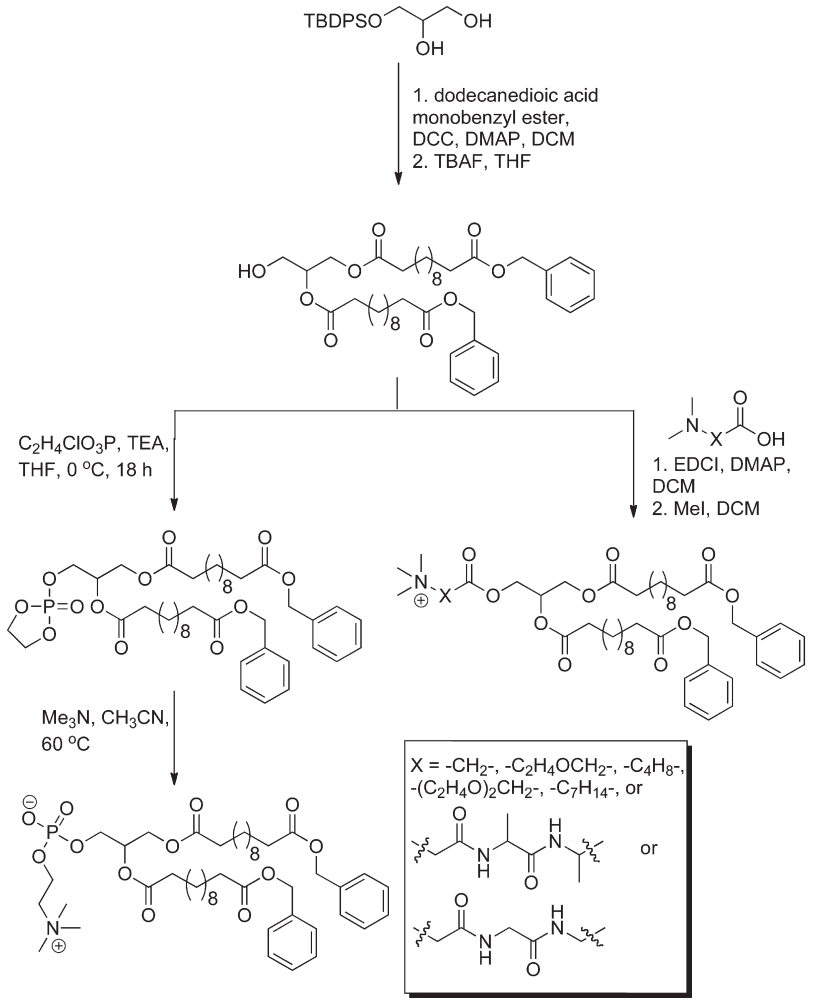


group, and subsequent carbodiimide facilitated formation of oleyl esters at 2′ and 3′ positions of the ribose ring affords the 5′-cationic nucleoside lipid.[22−24]

Conversion of 5′-OH of the 2′,3′-protected ribonucleosides to a dioxaphospholaneoxide moiety allows for a facile formation of a 5′-zwitterionic phosphate species following a reaction with trimethylamine (Scheme 4).[25−27] To form lipids, the 2′,3′-*O*-isopropylidene protecting group is subsequently removed and the two hydroxyl positions are converted into fatty acid esters using carbodiimide chemistry, affording zwitterionic lipids. Optionally, the zwitterionic phosphate lipids can be converted into cationic amphiphilic species by alkylation of the phosphate moiety using ethyl triflate, thus removing the negative charge from the molecule.

Starting from a 5′-protected commercially available phosphoramidite of deoxyadenosine or thymidine, the deoxynucleotide is converted to an anionic nucleotide-based lipid in three steps (Scheme 5).[28] We start with the addition of the appropriate alcohol in the presence of tetrazole, followed by oxidation of the phosphorus to the phosphate in the presence of iodine and removal of the nucleobase protecting

SCHEME 3. Synthetic Route to the Zwitterionic and Betaine Charge-Reversal Lipids



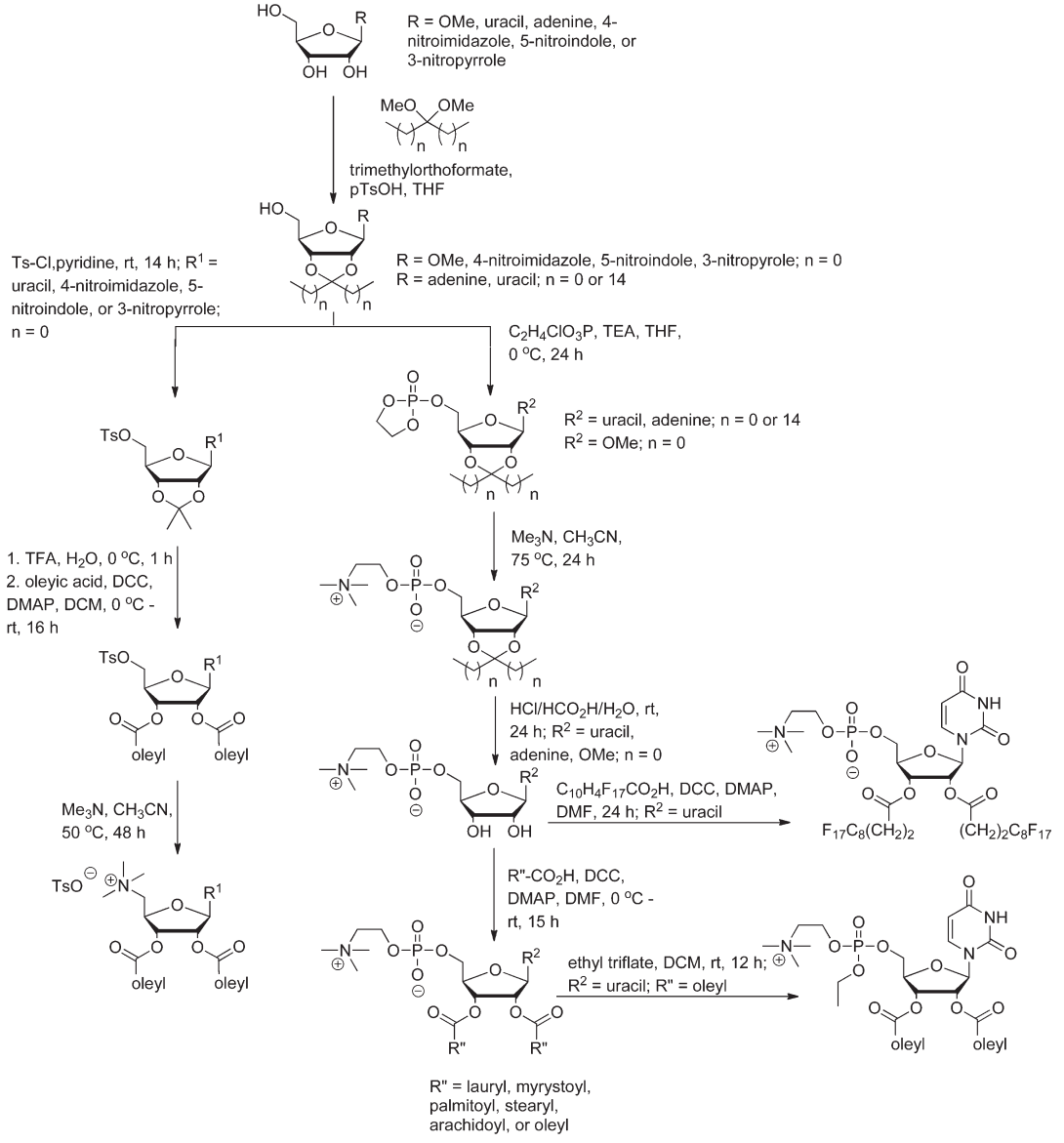
groups by NH_4OH (only in the case of adenosine). The subsequent deprotection of the 5′-OH is accomplished by trichloroacetic acid in DCM, affording the final nucleotide-based lipid.

3. Charge-Reversal Lipids for Gene Delivery

Our strategy entails using charge-reversal lipids that transform from cationic to anionic lipids intracellularly via esterase-induced hydrolysis (Figure 1).[17−20] These lipids belong to a family of functional, or stimuli-responsive, vectors, which includes amphiphiles that respond to reducing conditions, pH, enzymes, or temperature. For the charge-reversal lipids to perform as designed, they must undergo a series of reactions. First, the lipids must complex plasmid DNA and form a supramolecular DNA−cationic lipid assembly, known as a lipoplex. This lipoplex will ideally possess a net positive charge to facilitate cell uptake. Upon entry of the lipoplex into the cell, esterases hydrolyze the terminal ester linkages present in this assembly to afford anionic amphiphiles. Finally, the anionic amphiphiles repel DNA and destabilize the lipoplex, thus facilitating delivery of the plasmid DNA for subsequent transcription.

Consequently, we synthesized a family of charge-reversal lipids as shown in Figure 2.[17,18] Our top design candidate and prototype lipid, **1**, has a cationic ammonium headgroup to electrostatically bind DNA, lipophilic acyl chains to form a bilayer, and benzyl esters at the termini of the acyl chains for enzymatic hydrolysis and charge-reversal. This lipid was modeled after the well-known cationic lipid 1,2-dioleoyl-3-trimethylammonium propane (DOTAP). Furthermore, to assess the role of each structural component, we prepared a series of compounds **2**−**10**. Compound **2** is the hydrolyzed product of compound **1** and possesses an overall negative charge at neutral and slightly acidic (pH 5) conditions. Unlike lipid **1**, compound **2** does not bind DNA. As a result of the

SCHEME 4. Synthetic Route to the Cationic Nucleoside-Based Lipids



charged COO^- moieties at the ends of its hydrocarbon chains, **2** can destabilize lipid bilayers. Lipid **3** is designed to bind DNA; however, it cannot undergo the esterase-catalyzed reaction to yield an anionic amphiphile since it has terminal amide linkages. Compound **4** possesses a cationic charge but lacks the hydrophobic acyl chains also required for the formation of a stable lipoplex structure. Lipids **6**–**10** have various fatty acid chain lengths (C10:0, C12:0, and C16:0) and hydrolyzable end groups (benzyl, butyl, ethyl, etc).

Liposomes were formed using a lipid film hydration method, followed by sonication. These liposomes were then incubated with DNA in aqueous buffer and vortexed to form electrostatically bound lipoplex structures, which are then subjected to binding and release studies. Lipids **1**, **3**, and **5**–**10** bound DNA as determined by the ethidium bromide (EtBr) displacement assay. Of these cationic amphiphiles, all except **3**, **5**, and **7** released DNA in the presence of esterases. Amphiphile **3** possesses an amide, whereas the ester linkages present in amphiphile **5** (DOTAP) are deep within the hydrophobic structure, and thus, neither were cleaved by the enzyme. We hypothesized that analog **7**, which possesses a high T_m of 75 °C, compared with <50 °C for the other lipids, formed a stable lipoplex that inhibited the enzymatic hydrolysis reaction. The release of DNA from lipoplexes formed with **1**, **8**, and **9** occurs at varying rates, with lipids **1** and **8** being similar and lipid **9** being ~2× slower. Based on the dynamic nature of lipid asemblies, it is proposed that the endosomal esterases are

able to hydrolyze the terminal ester bonds during the flip–flop migration of the lipids in the bilayer.

Transfection experiments using the reporter gene β-galactosidase (β-gal) were performed with Chinese hamster ovarian (CHO) cells. Lipids **1**, **5** (DOTAP), and **6**–**10** all showed some level of transfection, but the efficiency was greatest for amphiphile **1** (Figure 3). The transfection efficiency of **1** was comparable to the commercial transfection reagent TransFast (Promega), a cationic lipid-based gene delivery vector. Cytotoxicity experiments performed showed negligible toxicity effects for all lipids studied (>80% viability in relation to the nontreated control) and comparable to that of the commercial reagent. Additionally, amphiphile **1** effectively transfected human cell lines with ~1.2× the transfection efficiency in HEK 293 cells and ~1.8× in K562 cells compared with TransFast.

Because lipid **1** showed the highest activity, we performed additional experiments to assess the structure that it forms with DNA. Transmission electron microscopy (TEM) of lipid **1** complexed with DNA showed the formation of lipoplex structures. The lamellar repeat period (d = 5.31 nm) and wide-angle spacing (0.46 nm) of the lipoplex structures, as determined by X-ray diffraction (XRD), indicated that they contained ordered bilayers. Control experiments of the lipids in the absence of DNA gave only slightly smaller lamellar repeat periods (5.22 nm), indicating that DNA interacts with the liposome surface to form complexes, as opposed to being sandwiched between bilayers, which is sometimes observed in lamellar lipoplexes (see sections below on betaine charge-reversal lipids and helper phospholipids). Lipid **1** formed liposomes

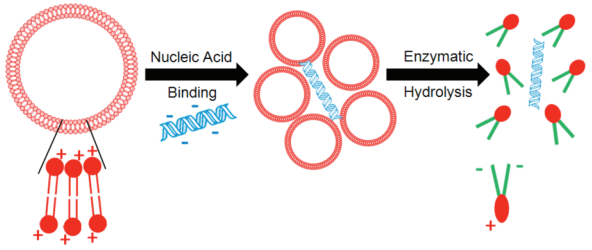


FIGURE 1. Schematic of the charge-reversal lipids transforming from a cationic to an anionic state by hydrolysis of the terminal ester linkages on the hydrophobic chains.

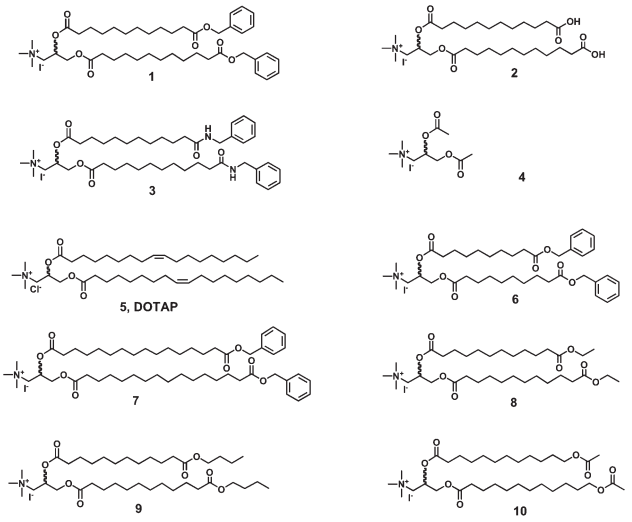
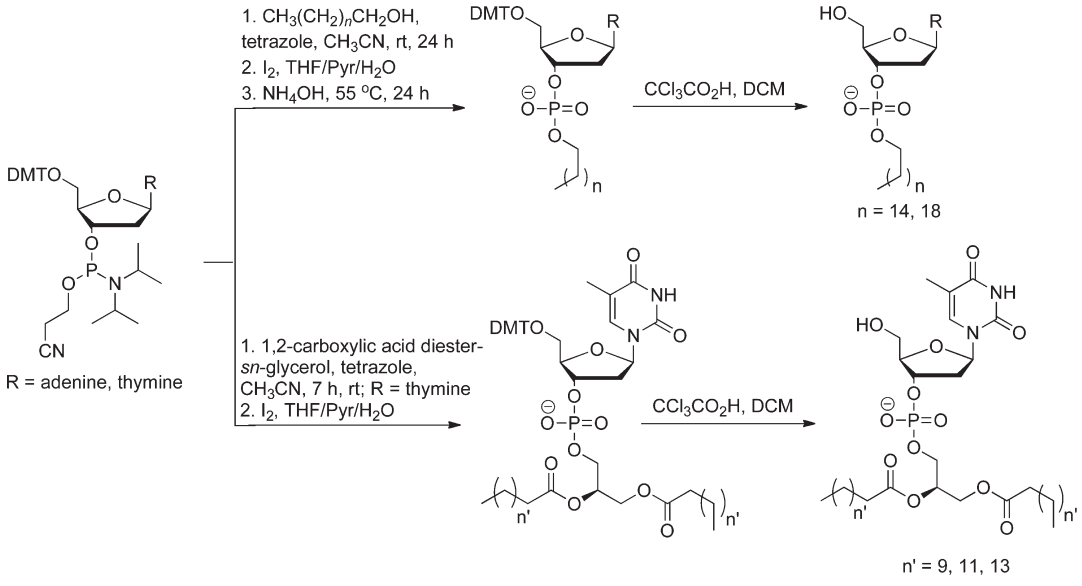


FIGURE 2. Charge-reversal lipids and their analogues, including DOTAP, under investigation for gene delivery.

SCHEME 5. Synthetic Route to the Anionic Nucleotide-Based Lipids



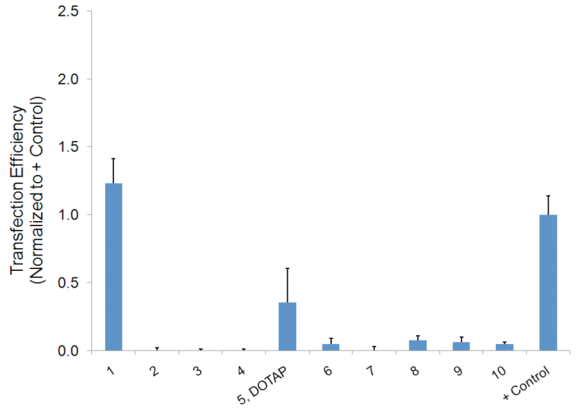


FIGURE 3. Transfection efficiency of the charge-reversal lipid, **1**, and its analogues in CHO cells. TransFast was used as the positive control. $N = 3$, avg + SD.

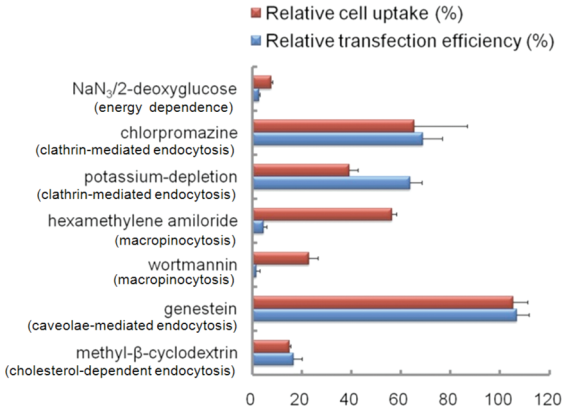


FIGURE 4. DNA transfection (blue) and cell uptake (red) in CHO cells in the presence of endocytosis inhibitors. Results are shown as relative to percent transfection (or uptake) without inhibitors. $N = 3$, avg + SD.

of ~110 nm in diameter as measured by dynamic light scattering (DLS), and the average size increased to ~562 nm when DNA was added.

With the above data collected, we next investigated the mechanism of uptake by the charge-reversal lipid, **1**.[29] Specifically, we carried out transfection assays in the presence of known pharmacological inhibitors that block particular cellular uptake pathways (Figure 4). The resulting cellular uptake and transfection activities showed that endocytosis was the pathway leading to transfection in CHO cells. When the macropinocytosis pathway was inhibited, β-gal expression was significantly impeded (95%). Meanwhile, the inhibition of the clathrin-mediated pathway only brought a 30% decrease in expression, and the inhibition of the caveolae-mediated pathway did not affect expression results. This conclusion was further corroborated by transfection kinetics and fluorescence colocalization studies, which supported fluid-phase uptake compared with receptor-mediated uptake.

We next synthesized and characterized a series of betaine charge-reversal lipids to evaluate the role of the spacer between the positive charge and the acyl chains. Lipids were designed with spacers of various lengths and flexibility, as shown in Figure 5.[20] All these lipids bind DNA and then release DNA in the presence of an esterase, as substantiated by an EtBr binding assay. The resulting lipoplexes formed with these betaine charge-reversal lipids range in size from 154 to 353 nm. XRD was used to further understand the supramolecular structures formed with DNA. All of the betaine lipids studied formed liposomes with lamellar repeat periods characteristic of lipid bilayers. Upon addition of DNA, only **B-GlyGly** complexes had a large enough increase in repeat period (1.8 nm) to indicate that the DNA was located

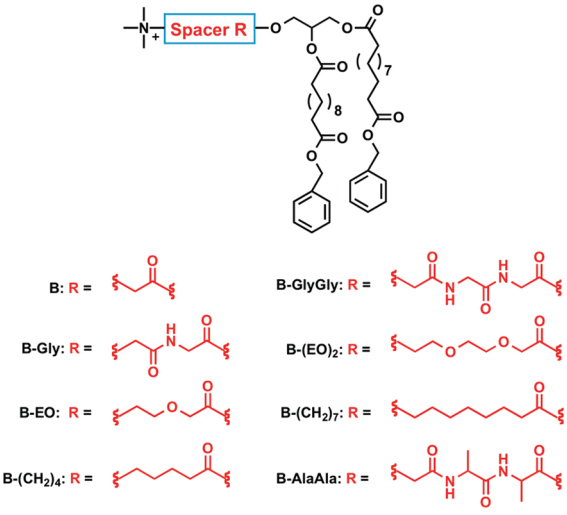


FIGURE 5. Structures of the betaine charge-reversal amphiphiles with different spacers.

between adjacent bilayers within the multilamellar liposomes. For the other betaine lipids, the repeat period increased less than 0.6 nm, indicating that the DNA only bound to the outer surface of the liposome and not between adjacent bilayers.

We next evaluated the ability of these betaine charge-reversal lipids to deliver DNA. DNA transfection experiments using β-gal, as before, were performed with CHO and NIH 3T3 (mouse embryonic fibroblast) cells. In CHO cells, lipids **B-Gly** and **B-GlyGly** showed the highest activity with transfection efficiencies ~1.25× and ~1.75× that of the positive control, Lipofectamine2000 (Invitrogen). The other lipids exhibited minimal transfection efficiency (less than ~0.65× that of the positive control). When testing **B-Gly** and **B-GlyGly** in NIH 3T3 cells, the resulting transfection was

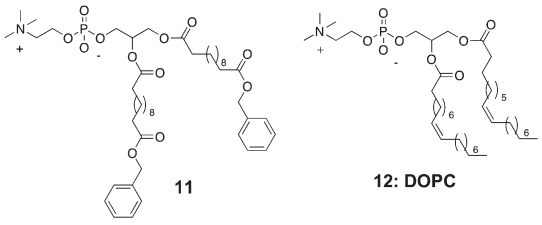


FIGURE 6. Structure of the charge-reversal helper lipid, **11**, and DOPC.

as high as ~0.24× and ~0.45× that of Lipofectamine2000, respectively. These results demonstrated that those lipids possessing hydrophilic and rigid spacers (i.e., **B-Gly** or **B-GlyGly**) were efficient vectors for DNA transfection. Viability assays performed with all amphiphiles showed no significant cytotoxicity (>85% viability, with the exception of **B-Gly** at high lipid ratios in NIH 3T3 cells). The charge-reversal effect was shown to be important in DNA transfection of CHO cells for these lipids by comparing the aforementioned results with those of **B-Gly-C$_{16}$** and **B-GlyGly-C$_{16}$**, saturated C16:0 analogues that lack the terminal ester group. These non-charge-reversal lipids had transfection efficiencies less than 0.1× those of their counterparts.

Finally, we extended this design element of the charge-reversal effect to the development of a functional helper phospholipid.[19] Helper lipids are added to cationic vector formulations to facilitate fusion of the bilayer with the membrane of the endosome and to improve transfection. Based on the structure of the widely used helper lipid dioleoylphosphatidylcholine (DOPC), the esterase-sensitive phospholipid, **11**, was composed of a zwitterionic phosphatidylcholine headgroup, two long acyl chains, and ester-linked benzyl terminal groups (Figure 6).

DLS studies showed that the vesicles formed from lipid **11** alone or mixed with DOTAP have average diameters of 106 and 127 nm, respectively. XRD patterns obtained from the **11**/DOTAP/DNA lipoplex (1:1 ratio of lipid **11** to DOTAP) samples gave a 1.5 nm larger lamellar repeat period than the liposomes, suggesting the formation of multilamellar structures with DNA sandwiched between adjacent bilayers in each repeating unit of the lipoplex. This structural arrangement is often observed in complexes formed from DNA–DOTAP and phosphatidylcholine.[30] At a lipid/DNA ratio of 20:1, β-gal transfection activity in CHO cells increased by 4.0× using **11**/DOTAP compared with DOPE/DOTAP.

4. Peptide-Based Lipids for Gene Delivery

The complexation of nucleic acids with the majority of reported lipids involves primarily electrostatic interactions between the positively charged lipids and the negatively

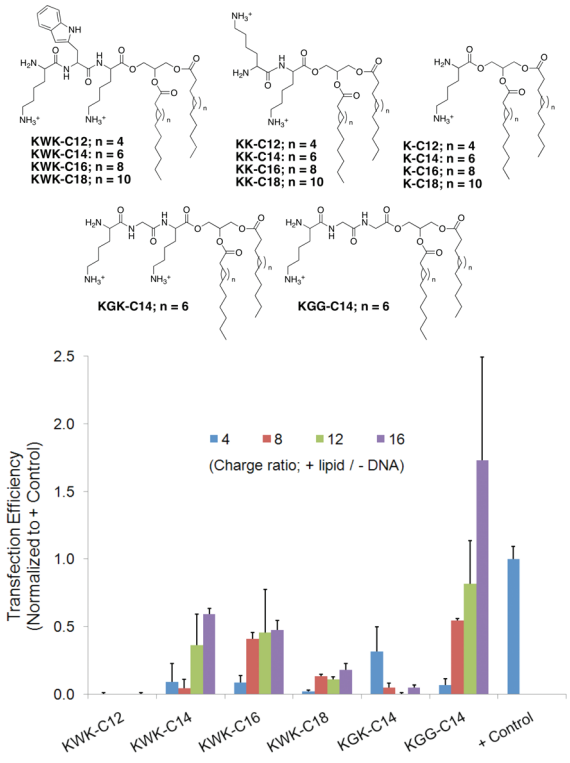


FIGURE 7. Structures (top) of the peptide-based amphiphiles synthesized. DNA transfection results (bottom) in CHO cells. Lipofectamine 2000 was used as the positive control. $N = 3$, avg + SD.

charged phosphate backbone of the nucleic acid. However, in biology, the recognition and binding of nucleic acids by proteins involves a collection of noncovalent interactions, not just electrostatics.[31] Examination of these protein–nucleic acid recognition motifs uncovers structures rich in aromatic and positively charged amino acids that provide important π-stacking and electrostatic contributions to the supramolecular structure. By designing a series of peptide-based lipids, the goal was to understand how these different interactions affect gene delivery outcomes.[21] A second motivation came from previous work with the charge-reversal lipid, **1**, which has poor aqueous solubility that limits formulation practices. We envisioned that a peptide headgroup could both increase solubility and improve complexation with DNA compared with a choline headgroup. Thus, we developed several new peptide-based lipids with varied headgroups and chain lengths (Figure 7).[21]

We selected the tripeptide Lys-Trp-Lys, KWK, as the headgroup for this study because both lysine and tryptophan are found in a number of structurally characterized DNA binding domains of proteins. The tripeptide itself, which possesses positive charges and an aromatic side chain, is known to bind DNA and has been used as a model to study protein–nucleic acid interactions.[32] We synthesized a series of lipids

where we varied both the headgroup (charge, aliphatic content, and aromatic content via the use of different amino acids) and the fatty acid chain length (C12:0, C14:0, C16:0, and C18:0) in order to determine their respective contributions to the delivery of DNA.

With regard to the supramolecular structure of these lipids and lipoplexes, we observed a trend among each type of headgroup in that longer chains resulted in an increase in lamellar repeat period. This was best characterized by the Lys-Lys (KK) dipeptide headgroup, such that repeat periods of 3.7, 4.1, and 5.1 nm were recorded for the C12:0, C14:0, and C18:0 lipids, respectively.[21] Upon incubation with DNA to form lipoplexes, these KK-based lipids did not show an increase in repeat period, indicating that DNA was not sandwiched within the multilayers. On the other hand, the tripeptide amphiphiles, **KWK-C14** and **KWK-C16**, had lamellar repeat periods that increased from 4.4 to 7.3 nm and 6.6 to 7.1 nm, respectively, when DNA was added to each of the systems. These data are consistent with a structural model where a bilayer phase is formed with the DNA chains located between the adjacent lipid bilayers within the multilamellar liposome.

Transfection assays were performed in CHO cells, again using β-gal. Several of these lipids are active transfection agents, as shown in Figure 7, with the Lys-Gly-Gly lipid, **KGG-C14**, performing the best and similar to the positive control, commercial transfection reagent Lipofectamine2000. Furthermore, in transfection experiments in NIH 3T3 cells, both **KWK-C16** and **KGG-C14** performed comparably to the positive control ($\sim$1.5$\times$ and $\sim$1.0$\times$, respectively). The tripeptide alone with no alkyl chains, **KWK**, did not induce transfection. These studies also showed that the mono- and dipeptide lipids do not transfect DNA efficiently. Additionally, the total charge is not the sole contributor to efficient transfection, and an aromatic residue capable of DNA intercalation is not required for improved delivery and transfection. Viability studies showed that in both CHO and NIH 3T3 cells, these lipopeptides were less cytotoxic than Lipofectamine2000 (viability >75% compared with the nontreated control), with the exception of elevated toxicity when using **KGG-C14** at charge ratios $\geq$12:1 in CHO cells (<40%) and toxicity with **KWK-C12** in NIH 3T3 cells (<50%).

5. Nucleoside-Based Lipids for Gene Delivery

Nucleoside-based lipids (nucleolipids) that have the base-pairing capabilities of nucleic acids and the self-assembly characteristics of lipids are ideal molecular candidates for the transport of oligodeoxynucleotides (ODN), DNA, and siRNA.[22−28,33−38] Again, we spotlight the importance of introducing different structural and molecular features into nucleolipids with the goal of enhancing transfection efficiency and minimizing cell cytotoxicity. With these nucleolipids, our focus is on the use of hydrogen bonding and π-stacking forces to favor the interactions between the lipids and the nucleic acids. For example, cationic nucleoside-based lipids composed of a cationic uridine lipid were synthesized and evaluated (Figure 8).[22,26]

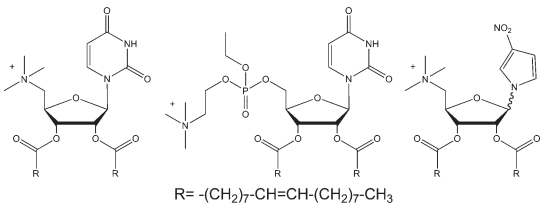


FIGURE 8. Chemical structures of uridine-based and 3-nitropyrrole nucleolipids used for transfection studies. (left to right) *N*-[5′-(2′,3′-dioleoyl)uridine]-*N*′,*N*′,*N*′-trimethylammonium, DOTAU; *O*-ethyl-dioleylphosphatidylcholinium-uridine, *O*-Et-DOUPC; 1′-(2′,3′-dioleyl-5′-trimethylammonium-D-ribofuranosyl)-3-nitropyrrole.

In vitro DNA transfection assays using a green fluorescent protein encoding plasmid (pEGFP) showed the efficacy of the nucleoside-based lipid *N*-[5′-(2′,3′-dioleoyl)uridine]-*N*′,*N*′,*N*′-trimethylammonium (DOTAU) as a transfection reagent (Figure 9).[22] Specifically in MCF-7 cells, DOTAU proved more effective at transfection than both Lipofectamine2000 and DOTAP. Meanwhile, DOTAU was only marginally effective at transfecting HeLa cells, as the positive controls performed better. *O*-Ethyl-dioleylphosphatidylcholinium-uridine (*O*-Et-DOUPC) was tested for transfection efficiency using a β-gal reporter assay and was shown to be as effective as the commercial transfection reagent TransFast and more efficient than DOTAP (Figure 9).[26] Importantly, the cytotoxicity of these nucleolipids, DOTAU and *O*-Et-DOUPC, was shown to be no greater than 20% in all cell types tested and at all charge ratios examined. It should be noted that these transfection experiments were performed in reduced-serum conditions, because transfection efficiency was impeded in the presence of serum. In parallel to biological studies, data collected from several experiments, including small-angle X-ray scattering (SAXS), indicated that DNA/nucleoside-based lipid complexes arrange into lamellar structures. In the case of the nucleolipid DOTAU, we found that lipoplexes were more compact than lipoplexes obtained with non-nucleolipid cationic reagents (DOTAP) due to tight associations between nucleolipids and DNA (repeat periods of 5.0 compared with 5.6 nm, respectively, when complexed with polyadenine ssDNA).

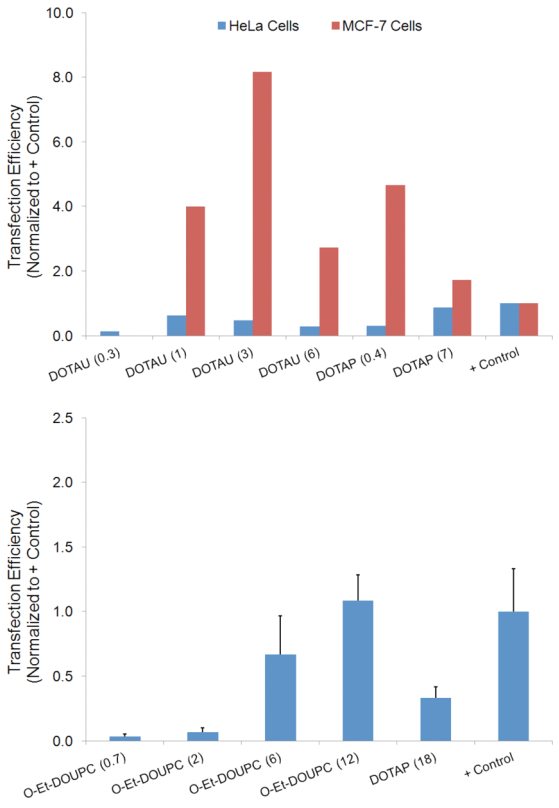


FIGURE 9. (top) pEGFP transfection in HeLa and MCF-7 cells using DOTAU, charge ratio in parentheses. Positive control is Lipofectamine2000. (bottom) β-gal transfection in CHO cells using *O*-Et-DOUPC, charge ratio in parentheses. Positive control is TransFast. $N = 3$, avg + SD.

Recently, cationic nucleoside lipids based on universal bases (3-nitropyrrole, 5-nitroindole, etc.) were synthesized from D-ribose. These were evaluated for siRNA delivery in order to promote complex formation, cellular uptake, and delivery of siRNA by using a nucleoside headgroup that would bind to all four bases, a universal interaction (Figure 8).[23,24] Physicochemical investigations revealed that these lipids form supramolecular organizations of nanometer size (132.4−516 nm dependent on stereochemistry of the 3-nitropyrrole nucleolipid and on the charge ratio of lipid/DNA).[23] The 5-nitroindole nucleoside lipid exhibited the highest binding affinity for siRNA and the greatest protein knockdown activities (similar to the positive control, NeoFX) on human liver carcinoma (HepG2) cells.[24] Additionally, the 3-nitropyrrole nucleolipids showed knockdown similar to NeoFX in HepG2 cells and showed improved knockdown (~2.0×) compared with NeoFX in NIH 3T3 cells. No significant cytotoxicity was observed for these universal-base nucleolipids (>90% viability relative to the nontreated controls, though increased cytoxicity occurred at charge ratios ≥20:1).

While recognizing the importance of the base-pair interactions, in an effort to expand the synthetic vector toolbox,

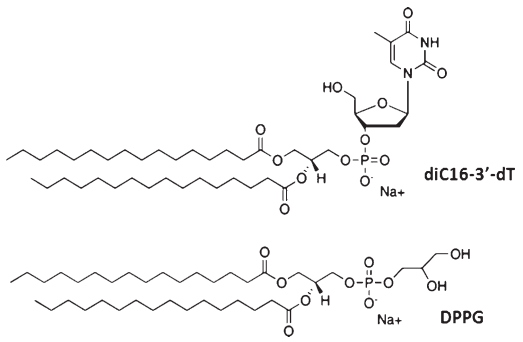


FIGURE 10. Chemical structure of an anionic nucleotide−lipid, the thymidine 3′-(1,2-dipalmitoyl-*sn*-glycero-3-phosphate) (**diC$_{16}$-3′-dT**), and a non-nucleotide lipid, DPPG.

we hypothesized that anionic or neutral nucleolipids might form supramolecular assemblies with nucleic acids and facilitate gene delivery. We first synthesized and evaluated anionic nucleolipoplexes composed of nucleotide-based lipids derived from 1,2-diacyl-*sn*-glycerol (**diC16-3′-dT**) and calcium (Figure 10).[28] Physicochemical studies demonstrated that stable nucleotide-based lipid/DNA complexes were not obtained at low concentrations of Ca^{2+} but only at concentrations of 1 mM Ca^{2+} or higher. Because this concentration is higher than intracellular calcium concentration (1 μM), a potential mechanism is present to release the DNA from the lipoplex inside the cells as the Ca^{2+} concentration equilibrium shifts. Transfection experiments carried out on human embryo kidney (HEK 293) cells showed that the nucleotide moiety enhances the transfection efficacy (1.6×) compared with the anionic phospholipid, DPPG. More recently we have extended the family of nucleotide-based lipids to acyclic nucleoside derivatives.[38] Transfection experiments performed on mammalian cells in the presence of Ca^{2+} revealed that the acyclic, anionic 1,2-dimyristoyl-*sn*-glycerol (**diC$_{14}$-T**) was as efficient as its cyclic analogue, **diC$_{14}$-dT**, at delivering plasmid DNA into cells. This indicates a minimal dependence of transfection activity on this structural component of the lipid.

6. Conclusions and Future Directions

Over the past seven years, we have designed, synthesized, and characterized over 50 new lipids for gene delivery applications. Our emphasis has been on improving transfection efficacy by focusing on chemical components that can be engineered and modified to vary the mechanism of nucleic acid binding, to alter nucleic acid-carrier release kinetics, and to enhance cellular uptake. By examining the challenges and successes of our predecessors, we have identified two main areas of interest: (1) using functional

lipids to induce changes in the supramolecular structure in order to control binding and release of the nucleic acid, and (2) designing bioinspired lipids to control and alter interactions with nucleic acids, to influence cell uptake, and to enhance lipoplex stability. Thus, our investigation of the charge-reversal, peptide-based, and nucleoside-based lipids enables us to address the various aspects of the hypotheses stated in the Introduction in order to develop better (and potentially clinically relevant) nonviral vectors.

Overall, our results highlight the sensitivity of gene transfection to subtle changes in chemical structure and the importance of identifying the optimized noncovalent interactions between the carrier and nucleic acid. We have learned from the charge-reversal lipids that a lipoplex system that switches from cationic to anionic affords greater transfection activity then a nonfunctional lipid of similar structure. By further altering these charge-reversal lipids by adding spacers between the cationic headgroup and the acyl chains, we discovered that rigid, hydrophilic spacers enhanced the delivery efficiency. This interest in structure–property–activity relationships continued with the peptide-based lipids, where we determined that tripeptide cationic headgroups conferred higher transfection activity compared with their mono- and dipeptide counterparts. The deliberate introduction of H-bonding and π-stacking interactions by incorporation of nucleoside headgroups also proved successful in transfection studies. Throughout this work, we also acknowledge the influence that alkyl chain length and lipoplex charge ratio (lipid/DNA) have on DNA transfection results and lipoplex structures formed.

Future research with these gene delivery vectors will continue to explore new methods to control binding and release kinetics of the lipoplexes (e.g., external stimuli-responsive lipids), while beginning *in vivo* work to evaluate gene therapy performance and to enhance delivery in cell lines that have been historically difficult to transfect (e.g., primary cell lines and stem cells). The next generation of lipids will incorporate headgroups and spacers that have proven successful in previous experiments. Characterization studies will focus on the structures formed both before and after complexation with nucleic acids and will attempt to link trends in lipoplex structure to cellular uptake and transfection. For the design of new nonviral gene delivery vectors, it is imperative to understand the current systems via determination of structure–property–activity relationships, as well as to examine and mimic the properties that make biological transfection systems so successful (e.g., viral transfection, surface binding peptides). The next decade of gene delivery and therapy will afford continued progress in the form of novel treatments and disease prevention aided by new lipid vector compositions and methods of their use.

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FOOTNOTES

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