Bioconjugate Chemistry

Review

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The Headgroup Evolution of Cationic Lipids for Gene Delivery

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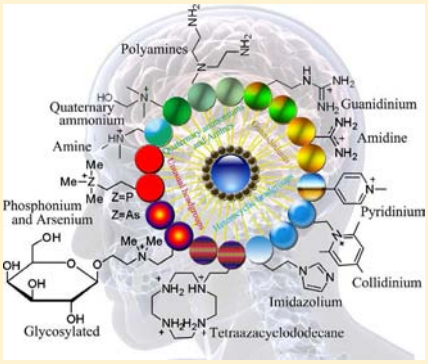
ABSTRACT: Cationic lipids are one of the most widely used nonviral vectors for gene delivery and are especially attractive because they can be easily synthesized and extensively characterized. Additionally, they can best facilitate the elucidation of structure−activity relationships by modifying each of their constituent domains. The polar hydrophilic headgroups enable the condensation of nucleic acids by electrostatic interactions with the negatively charged phosphate groups of the genes, and further govern transfection efficiency. The headgroups of cationic lipids play a crucial role for gene delivery; they can be quaternary ammoniums, amines, aminoacids or peptides, guanidiniums, heterocyclic headgroups, and some unusual headgroups. This review summarizes recent research results concerning the nature (such as the structure and shape of cationic headgroup) and density (such as the number and the spacing of cationic headgroup) of head functional groups for improving the design of efficient cationic lipids to overcome the critical barriers of *in vitro* and *in vivo* transfection.



INTRODUCTION

Gene therapy is generally considered a promising approach not only in the treatment of diseases with hereditary diseases, but also in the development of strategies for treatment and prevention of a broad variety of different acquired diseases such as cancer, degenerative disorders, and AIDS.[1−3] The most challenging issues for successful application of gene therapy to human diseases are the vectors used for delivery of the transgene into host cells. Broadly, depending on the vectors used for gene transfer, gene delivery is roughly divided into two main categories: viral and nonviral gene delivery.[4−6]

Viral vectors have been the favorites for several clinical applications due to highly efficient intracellular DNA delivery,[7,8] but their application is limited by host immune and inflammatory reactions, expense of production, potential to form replication-competent virions, size limit of the exogenous DNA (in the case of adeno-associated virus), and the risks of inducing tumorigenic mutations and generating active viral particles through recombination.[9−15] These limitations of viral vectors have prompted studies to find more efficient and flexible security system in the category of nonviral vectors.[4] Among the existing arsenal of nonviral vectors,[13,16−28] the distinct advantages associated with the use of cationic liposome include the following: simplicity of preparation, good repeatability and biodegradability, potential commercial value, and wide range of clinical application and safety.[29]

Cationic liposome-mediated gene transfer was one of the earliest strategies used to deliver exogenous genetic material into cells. In the early 1980s, some publications had reported the utilization of carrier systems for delivering exogenous globin mRNA, chromosomes, and DNA into host cells.[30] By 1987, Felgner and co-workers[12] first demonstrated the capability of delivering plasmids into living cells using cationic liposomes with unnatural glycerol backbone-based cationic transfection lipid-DOTMA (see Figure 1). Since this seminal work, a number of new cationic lipids have been synthesized and they are presently the most widely used constituents of nonviral gene carriers.[25−28,31−35] The early example of cationic lipid (1,2-bis(palmitoyloxy)-3-propyl) dimethyl(β-(*p*-nitrophenyl carbonato) ethyl)ammonium bromide, which consists of two saturated aliphatic chains (palmitic acid), ester linker, and quaternary ammonium group (see Figure 2).[37,38] Other lipids may contain different linkers such as the more stable ether linkages (e.g., DOTMA)[12] and more degradable carbamate linkages (e.g., DC-Chol (3β-[*N*-(*N*′,*N*′-dimethylaminoethane) carbamoyl]-cholesterol hydrochloride)),[39] and different hydrophobic parts such as the more rigide steroid hydrophobic domain (e.g., DC-Chol).[39,40] Some of these have been used in gene therapy clinical trials for treatment of cancer and other genetic disorders in the late 20th century,[31,32] while many others have become commercially available as transfection reagents for the transfection of cell lines in the laboratory. So far, cationic lipids as a component for gene delivery vehicles have become a major research tool for transferring exogenous genetic material into host cells.

Cationic lipids are amphiphilic molecules and generally consist of three parts: a hydrophobic domain (for example, aliphatic chains), a hydrophilic headgroup (for example,

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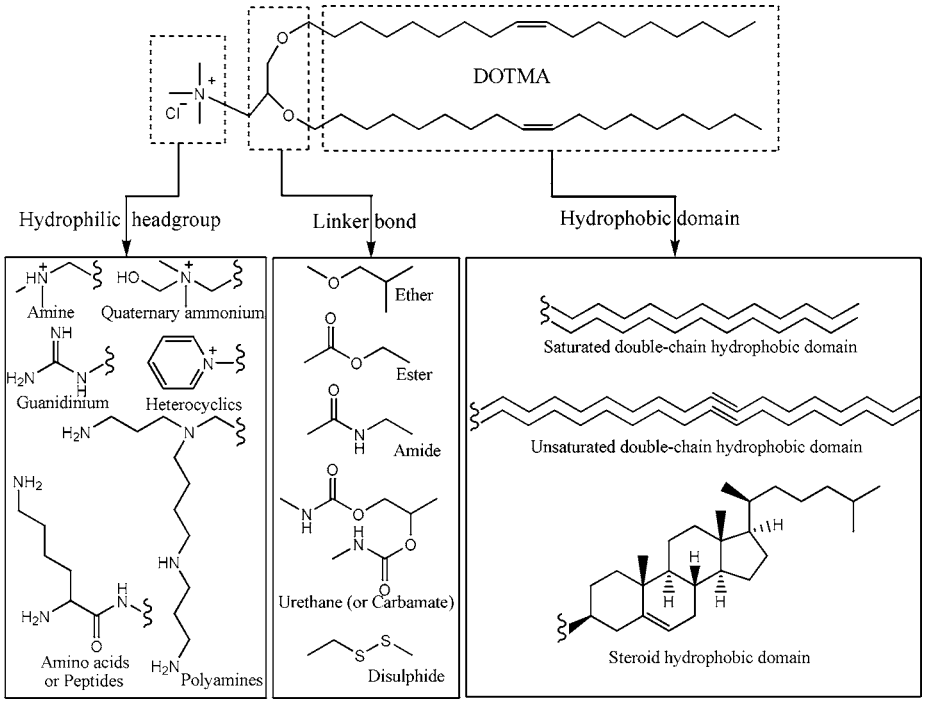

Figure 1. Representative structure of the cationic lipid DOTMA and examples of cationic lipid structural components: hydrophilic headgroup, linker bond, and hydrophobic domain.

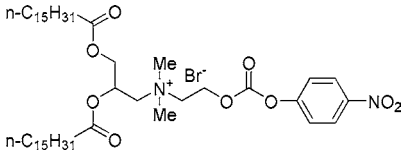

Figure 2. Early example of cationic lipids.

quaternary ammonium), and a spacer (including a linker bond (for example, ester bond) and backbone domain (for example, glycerol)) between these two parts (see Figure 1).[36] The hydrophilic headgroups exhibit one or more positive charges which trigger their interaction with negatively charged DNA through electrostatic attractions leading to the formation of complexes containing condensed DNA. Based on the structures of the hydrophilic headgroups, they can be grouped into six categories: quaternary ammoniums, amines, aminoacids or peptides, guanidiniums, heterocyclic headgroups, and some unusual headgroups, and for rational development, structurally modified in a systematic manner in order to correlate structure with transfection activity.

Despite the numerous studies that focus on structure–activity correlations of cationic lipids used in gene delivery, solid conclusions are rarely obtained.[41−43] This is mainly due to the complexity of the transfection pathway, and transfection efficiency can be affected by structural variations in the hydrophilic headgroups such as the charge density and the orientation in relation to the backbone, which can also affect toxicity of cationic lipids. We published a review article[29] related to the effects of the hydrophobic domains of cationic lipids on transfection efficiency. As a sister paper, this review discusses the evolution in the headgroups of cationic lipids and their influences on transfection efficiency, and hopes to provide suggestions on the development of cationic lipids through the discussion. The relationship between cationic lipid structure and toxicity in gene delivery is not discussed, because a comprehensive review on structure–toxicity correlations is available in the literature,[3] and amino acid and peptide headgroups are not included owing to a recent publication by us.[44]

STRATEGIES TO IMPROVE BINDING AFFINITY, CHARGE DENSITY, AND TRANSFECTION EFFICIENCY ON AMMONIUM AND AMINE HEADGROUPS

The amine headgroups often consist of primary, secondary, and tertiary amines or polyamine. Compared with the amine headgroups, quaternary ammoniums are by far the most frequently encountered. The relationships between the degree of hydration of the ammonium headgroup and the transfection efficiency and/or between the charge density of the ammonium headgroup and the transfection efficiency have long been a focus for concern and reform.[36,38]

Hydration Capacity and Charge Density of Cationic Lipids with Quaternary Ammonium Headgroup. Since the first cationic lipid bearing quaternary ammonium headgroup (DOTMA) was introduced by Felgner and co-workers in 1987, a number of cationic transfection lipids with quaternary ammonium headgroups (such as DOTAP, DDAB, CTAB) have been reported and found to be active in a wide variety of cell types (Figure 3).[12,36,43,45]

However, since these studies were conducted by many investigators using various cell lines and reporter systems, it is found that an important parameter that determines the transfection efficiency of lipids with quaternary ammonium headgroups is the presence of hydrophilic groups, more specifically hydroxyls. Accordingly, gene transfection by lipids DOTMA and the ester-linked variant DOTAP was modified by replacing the methyl group with the hydroxyethyl group to give DORIE (1,2-dioleoyl-3-dimethyl-hydroxyethyl ammonium bromide) and DORI (1,2-dioleoyloxypropyl-3-dimethyl-hydrox-

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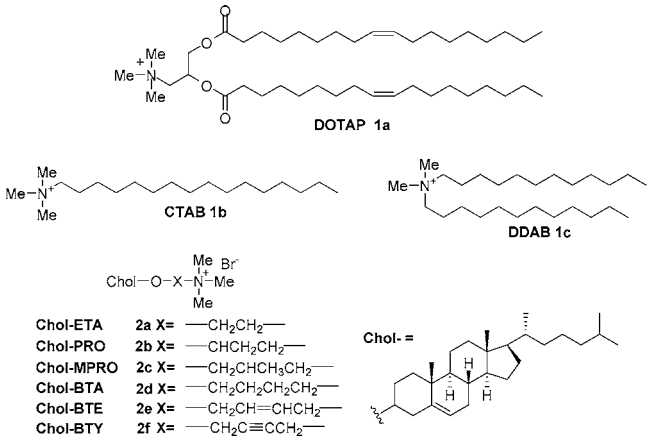

Figure 3. Chemical structures of cationic lipids with quaternary ammonium headgroups.

yethyl ammonium chloride), respectively (Figure 4).[13,46] DORIE or DORI containing hydrogen-bonding functionality

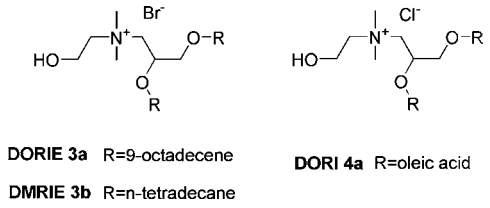

Figure 4. Chemical structures of DORIE, DMRIE, and DORI.

in the hydrophilic domain exhibited a corresponding decrease in observed lipid hydration. It was related to the greater ability of the functionalized cationic lipid to interact with cellular membranes via hydrogen bonding or by facilitating a greater electrostatic association, leading to greater activity than DOTMA or DOTAP *in vitro* and *in vivo*. When cationic lipids form an ion pair with DNA or with the phosphate moiety of DOPE (dioleyl phosphatidylethanolamine), some hydroxyl groups in the head of DORI or DORIE can participate in intermolecular hydrogen bonding, and interact with the aqueous phase, increasing the integrity of bilayer structure and stability of complexes. In our previous work, we synthesized a series of cationic lipids containing a hydroxyl moiety on the quaternary ammonium for liposome-mediated gene delivery, and some of them showed relatively higher transfection efficiency than the commercial transfection agents, Lipofectamine 2000 (Figure 5). It was suggested that the headgroup hydration could be decreased by the incorporation of a hydroxyalkyl chain capable of hydrogen bonding to neighboring headgroups, while it improved the compaction of DNA by several mechanisms: for example, DNA could form hydrogen bonds with the lipid, and the hydroxyl group could enhance the membrane hydration.[47]

Accordingly, a number of cationic lipids with quaternary ammonium headgroups containing one or more hydroxyl moieties have been synthesized and showed higher transfection efficiency.[48−54] Banerjee et al.[50] synthesized a series of nontoxic and nonglycerol-based cationic transfection lipids having one or more hydroxyalkyl chains on the quaternary ammonium headgroup. They found that, in the presence of an equimolar amount of cholesterol as the colipid, the transfection result of cationic lipid with two hydroxyethyl chains DHDEAB (**5b**) was 2−3-fold more efficient than that of DDAB (didecyldimethylammonium bromide) bearing two methyl groups directly linked to nitrogen instead of two hydroxyethyl groups. Compared with cationic lipid (**5a**) containing a single hydroxyethyl group in the headgroup regions, DHDEAB (**5b**) also showed higher transfection efficiency. Hydroxyisopropyl headgroup-based cationic lipids (**5c**) showed comparable transfection efficiencies to DHDEAB in COS-1 cells.[51] At the same time, they developed cationic glycolipids (**5d**) with four hydroxyl moieties in the headgroup regions, and found that the transfection efficiencies of these new lipids, when used in combination with cholesterol as a colipid, were observed to be higher than that of DHDEAB.[52] They have recently demonstrated that Tris-lipid **6a** (see Figure 6) containing a covalently grafted simple Tris-base component of the widely used biological Tris-buffer in the headgroup region was capable of imparting high serum compatibility and intravenous mouse lung transfection properties to cationic amphiphile, and it was likely to find future applications in nonviral gene therapy of inherited lung diseases.[53] They suggested that incorporation of two or more hydroxyl moieties in the headgroup appeared to convey decreased toxicity and increased transfection level.

Further, the group synthesized a series of cationic lipids (**7a−g**) that varied the chain length of the hydroxyalkyl moiety as shown in Figure 6, while keeping the remaining structure unchanged, they observed that the activity of lipid increased with a decrease in the hydroxyalkyl chain lengths.[55,56] It showed that transfection efficiencies of cationic lipids (**8a−9c**) with hydroxyalkyl headgroups were adversely affected by increased covalent distances between the hydroxyl functionality and the cationic centers.

Another important modification for the quaternary ammonium headgroups is the introduction of a second quaternary ammonium group which led to the formation of dimeric lipids

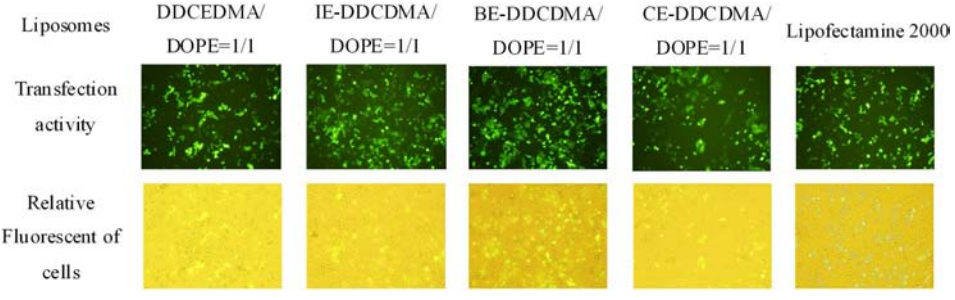

Figure 5. Expression of gene encoding for GFP transferred into HeLa cells by DDCEDMA/DOPE = 1/1, IE-DDCDMA/DOPE = 1/1, BE-DDCDMA/DOPE = 1/1, CE-DDCDMA/DOPE = 1/1, and Lipofectamine 2000.

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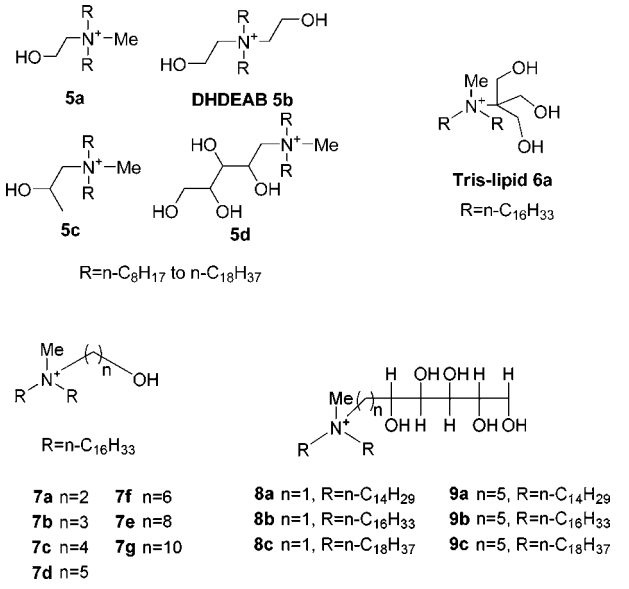

Figure 6. Chemical structures of quaternary cationic lipids with hydroxyl moieties.

(**10a**) (see Figure 7).[57−60] Rosenzweig et al.[58] found that diquaternary ammonium salts (**11a−13c**) constituted a new class of reagent for mediating transfection of DNA in mammalian cell lines, and these diquaternary ammonium salts exhibited good to excellent transfection activity in BHK cells. In a study, the dimer of *N,N*-dioleyl-*N,N*-dimethylammonium chloride (DODAC) joined by a hydrocarbon tether of six carbons in length (TODMAC6 **13c**) was shown to possess better transfection properties compared to DODAC. It appeared possible that the introduction of a second quaternary ammonium group could increase the strength of interaction with DNA. However, compared with TODMAC6 (**13c**), TODMAC3 (**13b**) with the hydrocarbon tether of three carbons in length exhibited lower transfection efficiency, behavior that was consistent with steric effects limiting the formation of ion pairs with anionic lipids.[61] Clearly, the transfection efficiency of dimeric lipids was controlled by the length of the spacer which determines headgroup charge separation and chain packing. Luciani et al.[62] also found that the length of the spacer influenced the complexation of DNA and the physicochemical features of lipoplexes (**14a−c**). Similar results in which the length of the spacer also affected the membrane-forming properties and the surface hydration of the membranes formed from these dimeric lipids (**15a−16e**) were obtained by Bajaj and co-workers.[63,64] In addition, they synthesized four novel cholesterol-based Gemini cationic lipids (**17a−d**) differing in the length of oxyethylene-type spacers [$-CH_2-(CH_2-O-CH_2)_n-CH_2-$] between each ammonium

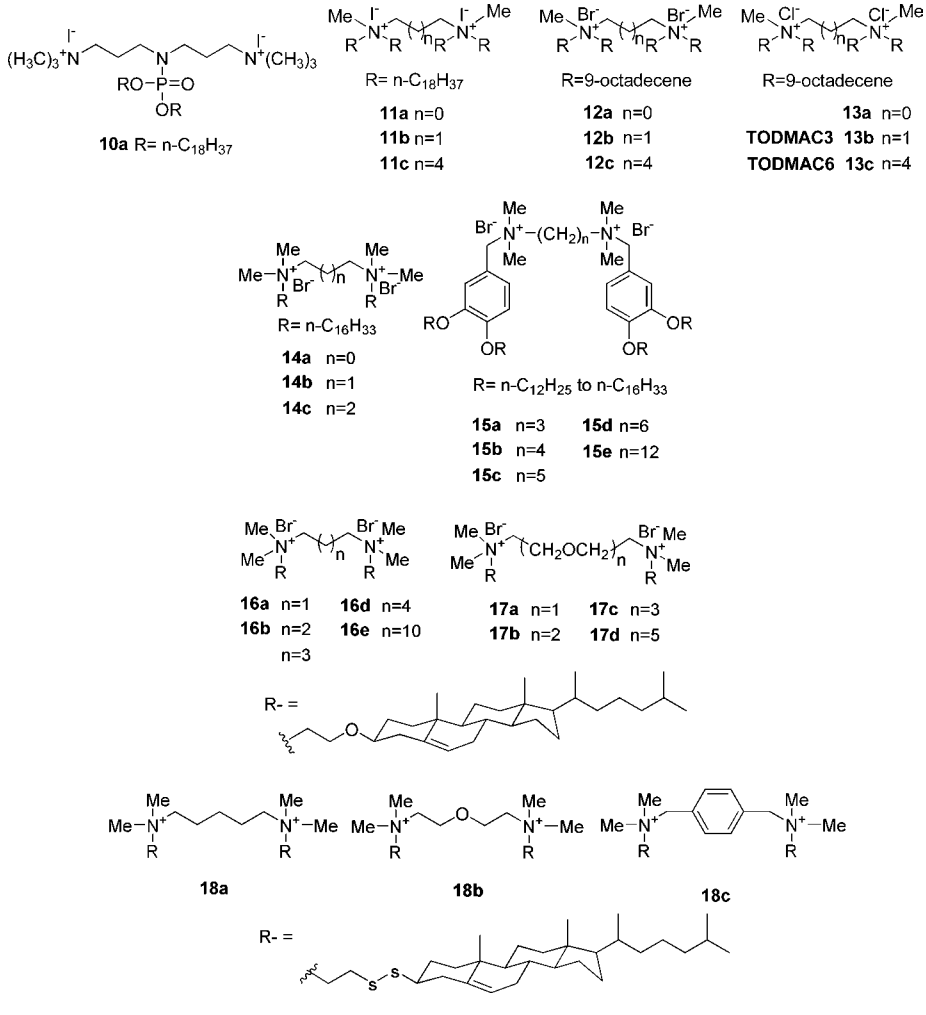

Figure 7. Chemical structures of dimeric lipids.

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headgroup, and found that incorporation of an oxyethylene spacer between the cationic ammonium headgroups could dramatically increase the transfection activities of the Gemini cholesterol lipids as compared to their monomeric counterparts.[65] Later, to further investigate the structure−activity relationship on cholesterol-based Gemini lipids differing in the length and the nature of the spacer between headgroups, they synthesized three Gemini disulfide lipids possessing hydrophobic flexible (−$(CH_2)_5$−; **18a**), hydrophilic flexible (−CH_2−CH_2−O−CH_2−CH_2−; **18b**), and hydrophobic rigid (−C_6H_4−; **18c**) spacer segments. The results showed that transfection efficacy of these lipids was dependent upon the cell line, as lipid formulations **18a** and **18c** were found to be effective in HeLa cell lines, whereas **18b** was found to be more effective than **18a** and **18c** in HT1080 cell lines.[66]

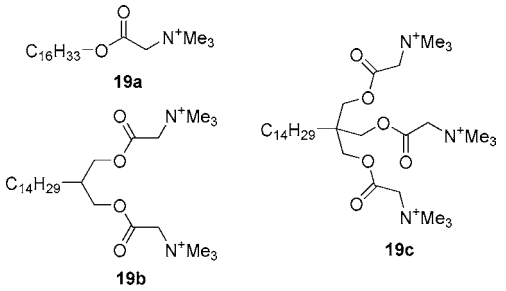

Figure 8. Chemical structures of multiheaded cationic amphiphiles.

Additionally, multiheaded cationic lipids bearing three or four quaternary ammonium headgroups have attracted more and more attention.[67−72] Although they were rarely used for gene delivery, they possessed important biological functions owing to their unique physical chemical properties, such as low micellar sizes and aggregation numbers and high CMCs (critical micellar concentration) and degrees of counterion dissociation.[67] A typical example of multiheaded cationic lipids was the research by Haldar et al.,[68,69] who synthesized and characterized a set of novel multiheaded cationic amphiphiles bearing one, two, and three trimethylammonium headgroups (**19a−c**). The antibacterial activity of multiheaded cationic lipids is significantly enhanced compared to that of single-headed cationic lipids, owing to their higher solubility in water and greater positive charge density per molecule, which enable them to interact better with the bacterial cell surface, leading to more efficient killing of the bacteria. This property renders this class of multiheaded cationic lipids potent candidates to replace their single-headed counterparts and also manifest effective gene transfection activity compared to their single-headed counterparts.[73]

In general, the modification to quaternary ammonium headgroups has two main pathways: the introduction of hydroxyls and the addition of more quaternary ammonium groups. The modification can affect the complexation and the release of DNA, the membrane-forming properties, and the surface hydration of the membranes formed from these lipids

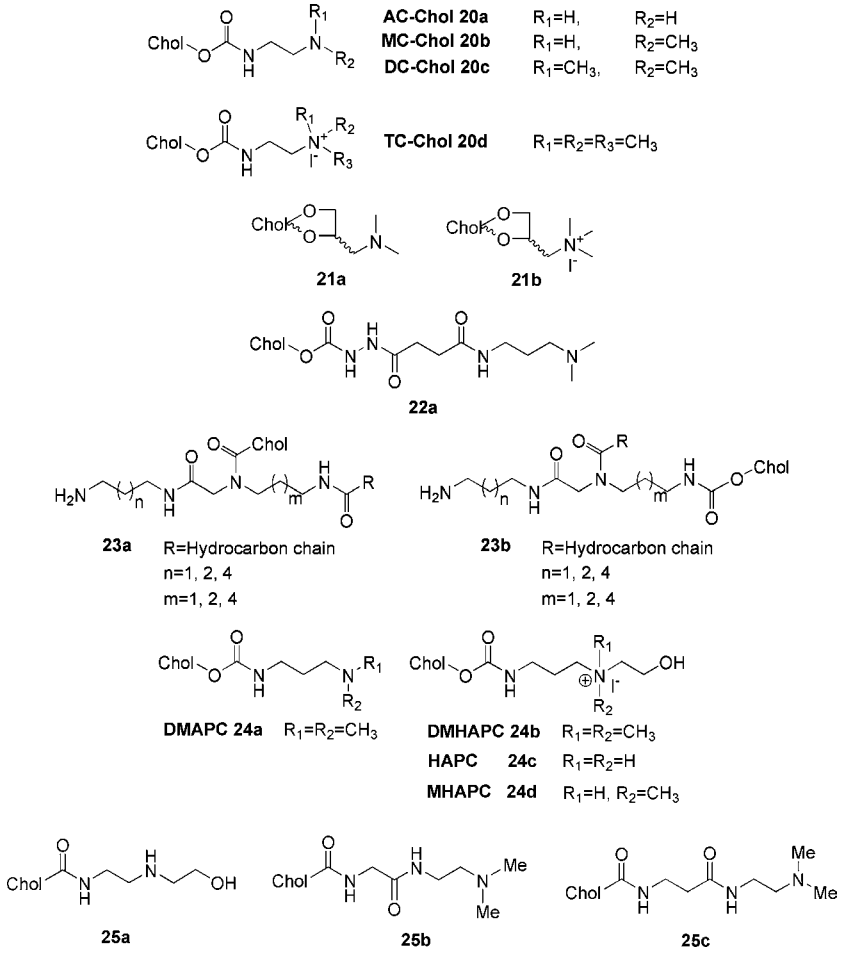

Figure 9. Chemical structures of cationic lipids with cholesterol moieties combined with the primary, secondary, or tertiary amino headgroups.

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and further lead to influencing the efficiency of gene transfection.

Neutral or Low Cationic Surface Charge Density of Cationic Lipids with Primary, Secondary and/or Tertiary Amine Headgroups. A quaternary ammonium group does not have any buffering function, since it is always charged at any pH; however, some lipids bearing a primary, secondary, or tertiary amino headgroups have better buffering function than ammonium counterparts. Amino derivatives of steroid lipids and DOPE are two known examples of such lipid compounds.[74−80]

The well-known commercially available DC-Chol (3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]-cholesterol hydrochloride, **20c**) with a tertiary amine headgroup linked to cholesterol was reported by Gao et al.[39] in 1991. Afterward, in order to estimate the effect of the cationic headgroup in the cholesterol lipids containing the urethane linker group, Kearns et al.[78] reported the synthesis and transfection potential of the primary amine analogue (3β-[*N*-(aminoethane)carbamoyl]-cholesterol (AC-Chol, **20a**), secondary amine derivative (3β-[*N*-(*N*′-methylaminoethane)carbamoyl]cholesterol (MC-Chol, **20b**), and the quaternary ammonium salt 3β-[*N*-(*N*′,*N*′,*N*′-trimethylaminoethane)carbamoyl]cholesterol iodide (TC-Chol, **20d**). They found that the most effective mediators of cell transfection were AC-Chol and MC-Chol, which elicited higher β-galactosidase activities and lower toxicities, in comparison to their tertiary and quaternary counterparts. Zhu et al.[79] showed that tertiary amine **21a** with cholesteryl groups as a hydrophobic tail gave higher transfection activity than that of quaternary ammoniums **21b**. It has been known for some time that the distance between the linker domain and the headgroup is an important factor in the design of vectors.[81] Some cholesterol-based cationic derivatives **22a−23b**, in which the amino headgroup and the hydrophobic moiety are separated by some atom spacers, have been designed and synthesized for gene delivery.[82,83] The results showed that cationic lipids displayed favorable transfection characteristics in selected human cancer lines when the amino headgroup and the hydrophobic tails were separated by a considerably greater distance (six or twelve carbon atom spacers).The replacement of a dimethylamino headgroup of the cationic cholesterol derivatives with a diisopropylamino or a diethylamino group showed significant decreases in transfection efficiency; in contrast, substitution of a dimethylamino headgroup with a hydroxyethylamino group exhibited higher transfection efficiency (**24a−25c**).[35,84,85] This suggested that the increased hydrophilicity of the amino headgroup of cholesterol derivatives increases the efficiency of gene transfection. Other steroid compounds used as hydrophobic moieties combine with the primary, secondary, or tertiary amino headgroups for cationic lipids including vitamin D[86] and cholesteryl,[87] which gave better transfection efficiency.

The well-known colipid DOPE with the ester linkage between the dioleyl chains and the primary amino headgroup (**26a**) often presents a super synergistic effect when used in cationic liposomes, because DOPE destabilizes lipid bilayers, and it has a high propensity to form reversed hexagonal phases at physiological or acidic conditions.[80] Some research showed that the primary amino headgroup (especially for amines in an unprotonated state) is incompatible in some cases with the ester linkage between the backbone and the hydrocarbon chains since the free base readily rearranges intramolecularly[88] or transfers intermolecularly[89] to the N-acyl analogue. The following approaches may solve the problem: (i) the amine should be stored in the salt form, such as TFA salts (**26b**);[88] (ii) the inclusion of another linker may result in the favorable intramolecular or intermolecular protonation with primary amino, such as phospholipids (**26c**).[90]

However, cationic lipids with the ether linkage between alkyl chains and primary amino headgroups are not faced with the same problems as mentioned above. Heyes et al.[91] synthesized a series of diether lipids bearing identical or different alkyl chains combined with primary amino headgroups (**26a**) and found that the shorter and more asymmetric diether lipids performed with higher transfection efficiency. Cationic lipids can also get better transfection and lower toxicity when another headgroup (such as quaternary ammonium salt) was introduced into the hydrophilic headgroup, by facilitating DNA and siRNA delivery into liposomes (**26d**).[90] Recently, Yingyongnarongkul et al.[92] designed and synthesized the primary amino cationic lipids with various spacer lengths and various hydrophobic tails (**26e**), and their results showed that cationic lipids with short spacer length and short hydrophobic tails bound to DNA and delivered DNA into HEK293 cells more efficient than those with longer ones. Results of the study were in line with the work by Mével and co-workers[93] who synthesized two new primary amino phosphoramidate lipids with various spacer lengths (**26f**).

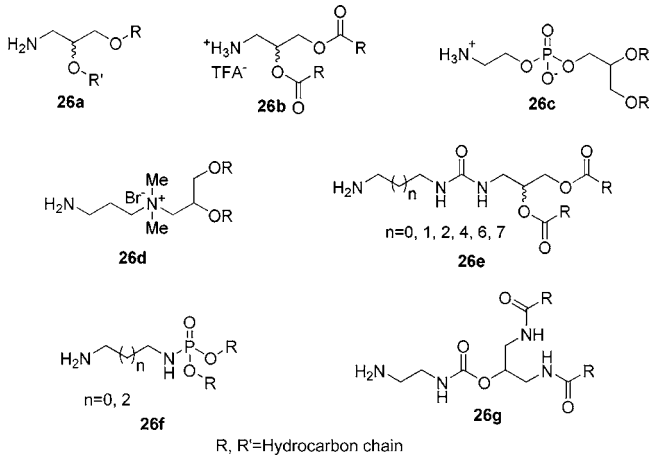

Figure 10. Chemical structures of cationic lipids with hydrocarbon chain moieties combined with the primary amino headgroups.

Compared with primary amino headgroup (**26g**), Savva and co-workers[94] found that the transfection activity of cationic lipid (**27a**) with tertiary amine polar headgroups was improved to a greater extent. However, cationic lipid whose hydrophobic domain comprised saturated double chains would increase the conformational disorder of the lipid, leading to a bilayer of increased fluidity. The inclusion of another ionizable tertiary amine headgroup may compensate for the increased width of the hydrophobic moiety and retain cylindrical geometry (**27b** versus **27a** and **27c**), which promoted assembly into lamellar structures. pDNA transported with the aid of the lipid containing bis-(2-dimethylaminoethyl)amine headgroups was present in a higher percentage inside cells when compared to plasmid delivered by the single tertiary amino counterparts.[95] However, an opposite result was obtained by Semple et al.[96] who recently adopted a rational approach to designing cationic lipids for use in formulations to deliver small interfering RNA (siRNA), showing that the dimethylamino headgroup (tertiary

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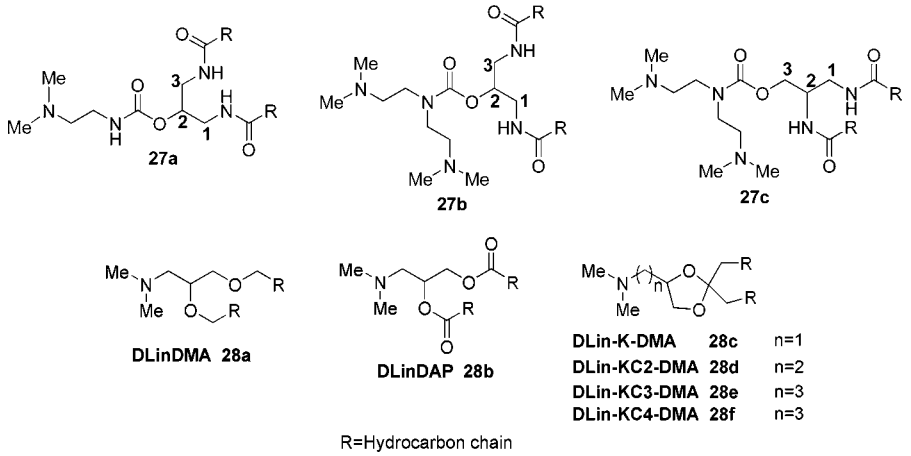

Figure 11. Chemical structures of cationic lipids with hydrocarbon chain moieties combined with the tertiary amino headgroups.

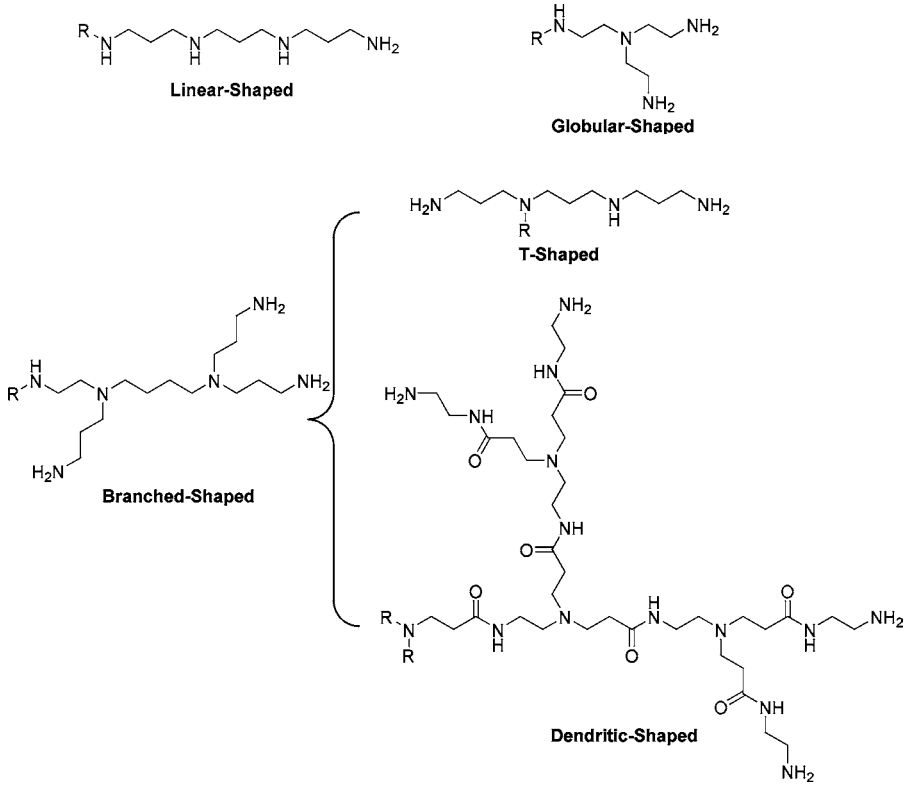

Figure 12. Shapes of the polyamine lipid.

amine headgroup) performed better than piperazino, morpholino, trimethylamino, or bis-dimethylamino counterparts. The amine headgroup should maintain a neutral or low cationic surface charge density at pH 7.4 because of crowding with the neighboring groups, providing longer half-life in the circulation, and reducing nonspecific cytotoxicity. The best-performing lipid, DLin-KC2-DMA (**28d**), which was produced by inserting a single additional methylene group into the headgroup of DLin-K-DMA (**28c**), was well-tolerated in both rodent and nonhuman primates and exhibited *in vivo* activity at siRNA doses as low as 0.01 mg/kg in rodents, as well as silencing a therapeutically significant gene in nonhuman primates.

To summarize, cationic lipids bearing primary, secondary, and/or tertiary amine headgroups usually have the neutral or low cationic surface charge density at pH 7.4, which could be changed by the introduction of another amine headgroup or altering the spacer lengths and linker, thus resulting in improvement of their transfection efficiency.

Polyamine Lipids Modified with Shape Manipulation. Polyamine lipids are generally multivalent and they are expected to form liposomes with a greater surface charge density than the monoamino equivalents. Generally speaking, polyamine lipids are considered better than the latter at DNA or siRNA binding and delivery to the target cells, and it is possible that the presence of protonation sites with different pK_a values (generated by polyamine) may lead to buffering of the endosomal acidification, thereby protecting the DNA from degradation and providing a possible endosome escape mechanism. In a systematic study of the roles of parameters (including hydrophobic domain, the structure of headgroup

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and the shape of lipopolyamines), the shape has greatly impact on transfection efficiency. Based on the relative orientation of the hydrophobic chain and the headgroup, the shape of the polyamine lipid generally contains three types: linear-, globular-, and branched-(including dendritic- and T-shaped) shapes (see Figure 12).[36]

Linear-Shaped Polyamine Headgroup. Linear-shaped polyamine compounds are generally prepared from the modification of suitable polyamines,[36] such as putrescine, cadaverine, spermidine, norspermidine, spermine, norspermine, and caldopentamine (see Figure 13). Recently, some convenient synthesis methodologies for the production of cholesterol-based polyamines have also been developed, such as solid-phase methodology.[97−101]

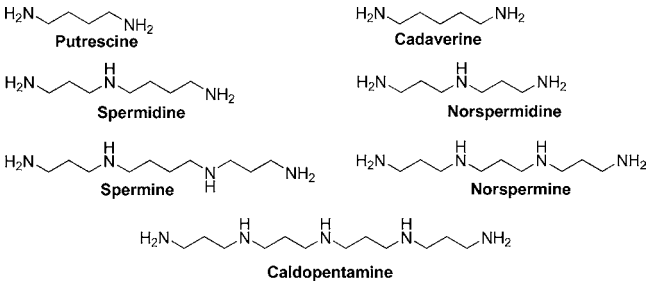

Figure 13. Chemical structures of polyamines.

A typical example has been found by Cooper and co-workers,[97] who reported the synthesis of polyamine carbamates of cholesterol from N-alkylate amino alcohols and cholesterol chloroformate. Although the number of amine functional groups (**29a−32a**) is very important in transfection efficiency of the linear polyamine, the methylene spacing almost appears to be a more critical factor in promoting efficient gene delivery than the absolute number of amine functional groups. It is possible that liposomes containing such unnatural polyamines are able to interact with DNA more tightly than DC-Chol/DOPE cationic liposome so as to promote efficient gene transfer across the outer cell membrane, but not so tightly that they are unable to release the DNA into the cell cytoplasm after transfer has taken place. Then, Geall et al.[98] showed that transfection efficiency of cationic lipids with polyamine (this section is not for ammonium) headgroups increased with the increase in the spacing (3.2.3 < 3.3.3 < 3.4.3). This trend may be attributed to increase in net cationic charge at pH 7.4 of the cationic lipids with longer spacer lengths. Additionally, for the number of amine functional groups, cationic lipid bearing three amine functional groups (**30e**) exhibited higher transfection efficiency than that with five amine functional groups (**32a**). It is possible that **30e** is able to better distribute the charge density in comparison to **32a** in the same net charge at pH 7.4.

The transfection activity of linear-shaped polyamine lipid is also influenced by a linker type and the amount of cholesterol residues except the length of the linear polyamine headgroup (**33a−35a**). R. Sheng et al.[102] demonstrated low cytotoxicity, strong pDNA binding affinity, high transgenetic efficacy, high intracellular uptake capability, and specific cellular localization of pDNA at the periphery of cell nuclei for newly prepared CHOSS lipid (**35a**). Its cholesterol group was bonded to cationic headgroups via disulfide linkage, which may pave a new way to utilize cholesterol, amino acids, and other functional natural products to prepare efficient gene/drug delivery carriers with simple structure and low cytotoxicity. A few examples showing transfection efficiency of polyamine lipid containing two cholesterol units, carbamate linker, and spacer of six methylene groups (**34a−c**) significantly exceeded that of other analogues tested and Lipofectamine 2000.[103] Additionally, Bajaj et al.[104] synthesized nine lipopolymers based on low molecular weight polyethyleneimines (PEI) and cholesterol via an ether linkage between the polymer amine and the cholesterol backbone. PEI-cholesterol-based lipopolymers possessed serum compatibility and could effectively deliver genes into cells even in the presence of high percentages of serum. Similarly, bis-substituted compound **36e** which was synthesized via direct amide coupling of spermine to the C-24 position of cholic acid analogues combined with its single-substituted analogue led to enhancement in transfection activity.[27]

It is known that the membrane fluidity increases when the cholesterol of linear-shaped polyamine lipids is replaced by aliphatic chain, by disrupting membrane packaging and facilitating DNA escape inside the cells; thus, these lipids (**37a−c**) also showed higher transfection efficiency.[105−109] Byk et al.[108,109] prepared a series of linear lipopolyamines (**38a−c**) that were 5−10 times more effective at transfection than those with other shaped polar domains in HeLa cells. Afterward, a homologous series of lipophilic polyamines (**39a−g**) were synthesized by Gardner and co-workers,[110] who found that the number and position of the positive charges along the polyamine scaffold played a key role in DNA delivery and in determining the transfection efficiency. Recently, a series of spermine-based linear cationic lipids (**40a−e**) were more efficient for binding to and delivering pDNA and siRNA with high cell viability even in a primary skin cell line than branched series (**41a−h**) which have been reported by some groups.[111,112]

Globular- and Branched-Shaped Polyamine Headgroup. To obtain novel tools for increased *in vitro* and *in vivo* gene delivery, many investigations aim at implementing a step by step strategy by systematic modifications into the headgroup domain. The introduction of globular- and branched-shaped polyamines into headgroups is an important variation.

Although globular-shaped polyamine lipids are able to achieve transfection, they display lower transfection activities as compared to the linear- or branched-shaped polyamine derivatives. The results show that the shorter ethylene chains between the amines in the globular lipids probably have an impact on the total positive charges available for binding to DNA at physiological conditions compared with propylene or tetramethylene in polyamine derivatives. Therefore, it can cause, in part, decreased pK_a values of the different amines in the globular compounds.[108]

In addition to globular-shaped polyamine headgroups, lipids with branched-shaped polyamine headgroups have exhibited significant gene transfer as compared to various commercially available agents. Some groups were acylated at the positions of N^4 and N^9 of the N^1,N^{12}-diamidinospermine, respectively, to synthesize branched-shaped polyamine lipids (**41a−h**), and found that N^4,N^9-dioleoyl spermine (**41g**, commercially available as LipoGen) and N^4,N^9-distearoyl spermine (**41f**) could efficiently condense pDNA and achieve the highest transfection levels with the highest cell viability among the studied lipopolyamines even in the presence of serum.[113−116] Additionally, a branched-shaped polyamine cationic lipid (**42a**) bearing a headgroup that is based on the amino acid ornithine and loaded with five charges in the fully protonated state

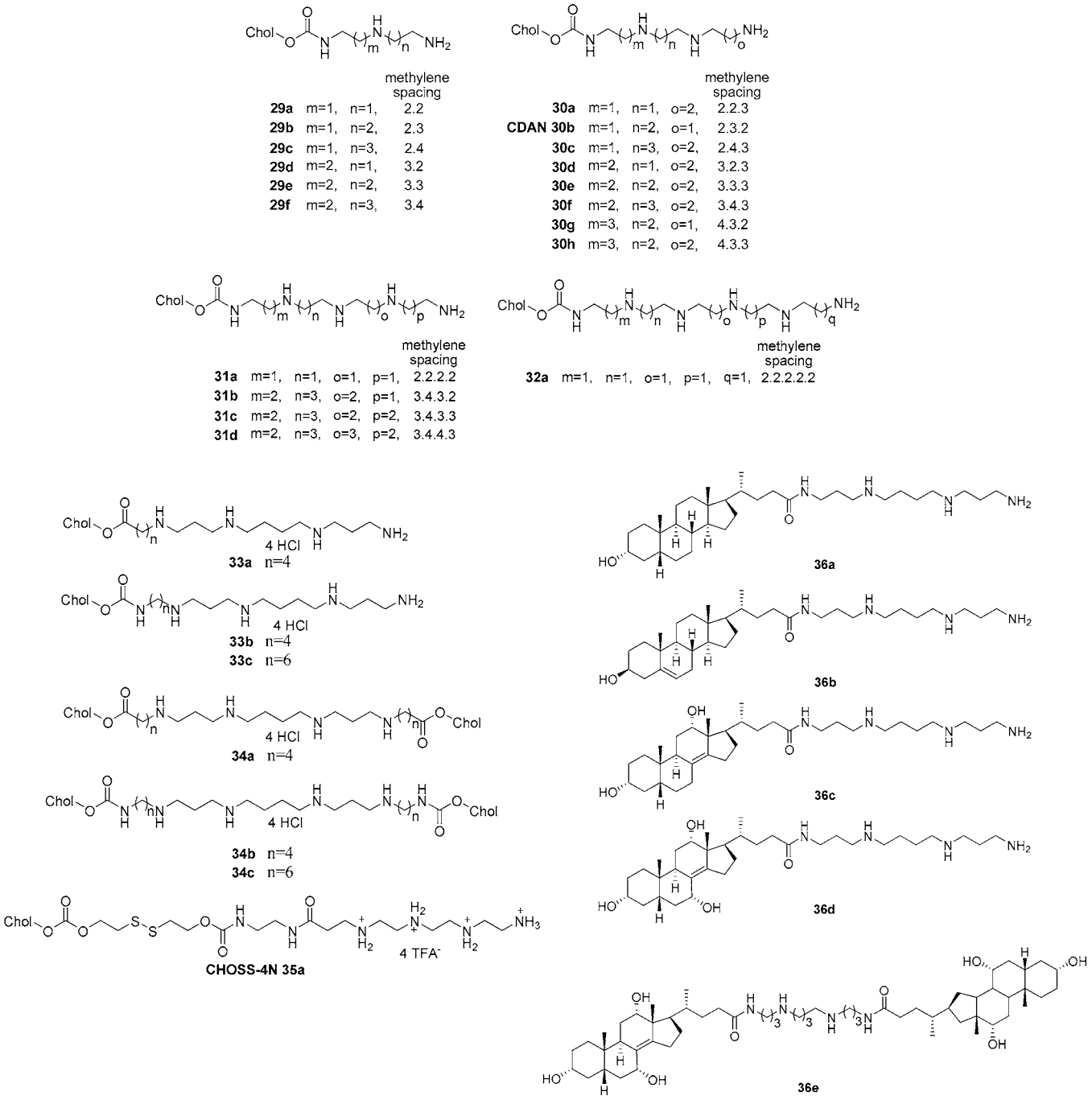

Figure 14. Chemical structures of linear-shaped polyamine lipids bearing cholesterol.

exhibited higher transfection efficiencies for both DNA and siRNA versus their monocharge counterparts.[117−119] As it was not biodegradable, some cleavable bonds (for example, disulfide bond) were introduced into the linker to degrade the polyamine cationic lipid (**42b−e**).[120] The results demonstrated that degradable disulfide spacers could be used to reduce the cytotoxicity of synthetic nonviral gene delivery carriers without compromising its transfection efficiency.

In polyamine branched-shaped lipids, dendritic or T-shaped lipids are usually used for research.[121−123] Some dendritic systems showed high-affinity DNA binding at low nanomolar concentrations as a consequence of its multivalent, biologically derived spermine ligands.[124] Takahashi et al.[121] designed a series of cationic lipid (**43a−d**) with a cationic polar group in the polyamidoamine dendron and two dodecyl chains, which can transfect cells efficiently by synergy of the endosome buffering and membrane fusion with endosome. They found that the buffering capacity of the dendron-bearing lipids might be related to their transfection activity since the transfection activity of the lipoplexes of the dendron-bearing lipids increased in the order of their buffering ability (this is consistent with the order of the number of tertiary amino groups in these molecules (**43b** < **43c** < **43d**). Then, Ewert and co-workers[122] have synthesized new polyamine cationic lipids with highly charged dendritic headgroups that were constructed from ornithine cores and ornithine or carboxyspermine end groups. These dendritic lipids generally showed high transfection

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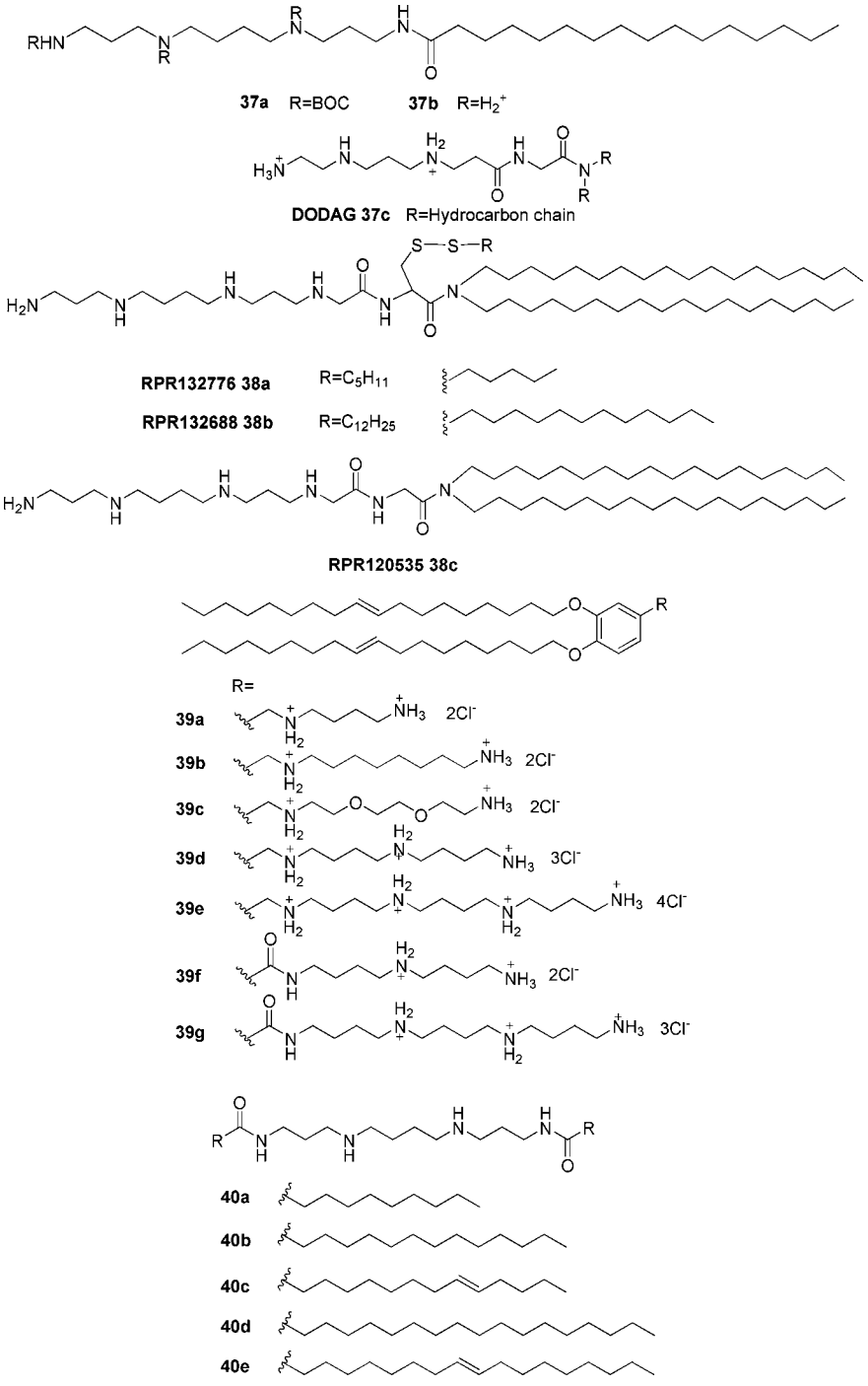

Figure 15. Chemical structures of linear-shaped polyamine lipids bearing hydrocarbon chain.

efficiency over a broad range of composition and low cytotoxicity *in vitro*.

A T-shaped lipid, where the lipid domain is attached in the middle of the polyamine chain, exhibited considerably higher level of transfection as compared to lipids with linear polyamine headgroups.[97,98,125] DOGS (**44a**) and DOSPA (**44b**) are two early representative examples of such lipid compounds with aliphatic chains. Other known examples include DPPES (**44c**), $(C_8)_2$Gly Sper^{3+} (**44d**), and $(C_{18})_2$Sper^{3+} (**44e**).[126] They have a functionalized T-shaped polyamine headgroup that confers the ability to act as a buffer, which could extend the amount of time needed to activate acid hydrolases; thereby, it may be able to explain why some multivalent cationic lipids can exhibit higher transfection efficiencies compared with their monovalent counterparts.[127] After studying a series of linear-shaped polyamine lipids (RPR family **38a−c**), Tranchant and co-workers[128] focused interest on the T-shaped lipopolyamine and found that luciferase expression was higher when plasmid DNA was complexed with T-shaped polyamine lipids RPR209120/DOPE (**44j**, see Figure 18). However, K. Fabio et al.[129] described the synthesis of new perfluorinated dimerizable detergents containing a T-shaped polyamine head (**45a−e**). Although these dimerizable perfluorinated spermine-based detergents can form small-sized cationic monomolecular DNA nanoparticles (<40 nm), they proved to be poor nonspecific transfection agents *in vitro*, even in the presence

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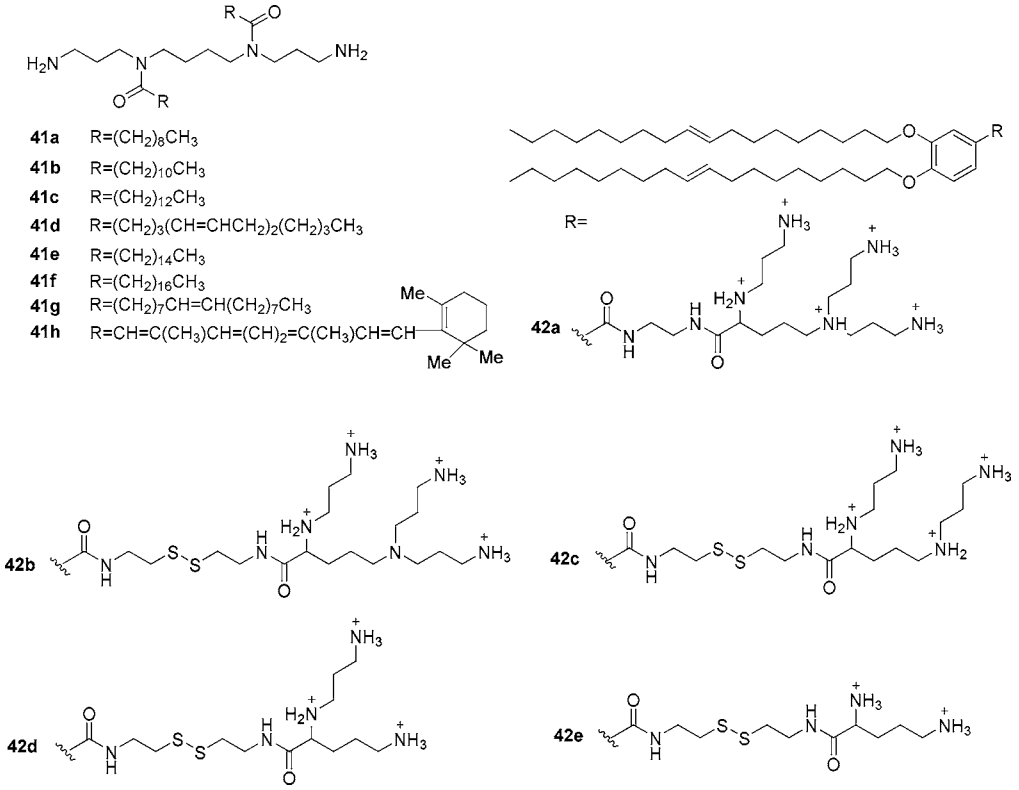

Figure 16. Chemical structures of branched-shaped polyamine lipids.

of chloroquine. It is probably because Brownian motion prevents these very small particles from sedimentation and adsorption onto the adherent cell monolayer, and, consequently, from proteoglycan-triggered endocytosis.

Cationic lipid GL-67 (**46a**, see Figure 19) is an example of cholesterol-based cationic lipids bearing T-shaped headgroups. It has been found to be particularly efficient for gene transfer to cultured cells and in murine lungs compared with the rest of cholesterol and double chain derivatives, including the parent compound, DC-Chol (**20c**).[130] Since then, other T-shaped derivatives (**47a**–**r**) also showed higher efficiency than those of other shapes (such as dendritic- and globular-shaped) and some commercially available lipids. Some cholesterol-based cationic lipids bearing T-shaped headgroups were synthesized by parallel solid-phase chemistry,[131] which exhibited high transfection efficiency. T-shaped polyamines resembled a cognate ligand for a cell-surface receptor, and thus the polyamines were better at promoting effective interactions with the pDNA or facilitating attachment and entry into target cells.

Cationic lipids containing polyamines have pH-buffering functions, which are generally expected to be better at DNA binding compared with primary, secondary, and tertiary amines and quaternary ammonium salts and may inhibit the early endosomes from maturing and delay the fusion between endosomes and lysomes, thus increasing the stability of plasmid DNA in cells since acidic lysomes are where most substances are destroyed. Linear-shaped polyamine lipids seem to be generally more effective than those with other shaped polar domains, but they can automatically fold themselves in the formation of liposomes, leading to incomplete DNA packaging. Branched-shaped polyamine lipids have the advantage of avoiding the problem and can include additional protonation sites without affecting DNA binding. Based on the experimental study on lipopolyamines and their variants, the T-shaped polyamine lipids exhibited higher transfection efficiency compared with others. Despite the improved DNA binding of polyamine headgroups, the cytotoxicity of these compounds stimulates the investigation of alternative functionalities, including cleavable bonds and the spacing and the number of amine functional groups.

DEVELOPMENT OF CATIONIC LIPIDS BEARING GUANIDINIUM FUNCTIONALITY

Guanidine headgroups have been proposed to be incorporated into cationic lipids, which are very efficient at compacting DNA and delivering it into cells.[34,93,113,132−135] Additionally, guanidine is found naturally in arginine amino acid residues which play a key role in DNA-binding proteins such as histones and protamines.[136] Guanidine headgroups of cationic lipids generally contain either monoguanidinium (monovalent) or conjugates of guanidinium and other headgroups (polyvalent). A common variation is the use of derived guanidines, such as amidines[137,138] and cyclic guanidines.[139]

Herscovici et al.[140] reported the synthesis of cationic lipids (**48a** and **48b**) with a hydrophobic domain which was connected to a guanidinium entity by an unsaturated glycoside scaffold, and these lipids can significantly compact DNA and form colloidally stable lipoplexes. Then, Mével and coworkers[93] prepared a series of new cationic phosphoramidate lipids (**49a** and **49b**) with chemical similarity to cell membrane phospholipids and a guanidinium polar headgroup, and they found that the transfection efficiency was better when the spacer between the phosphoramidate moiety and the polar head was short.

The introduction of other groups (e.g., pyridinium (**50a**) and amine headgroups (**50b**–**51b**)) to the monoguanidinium

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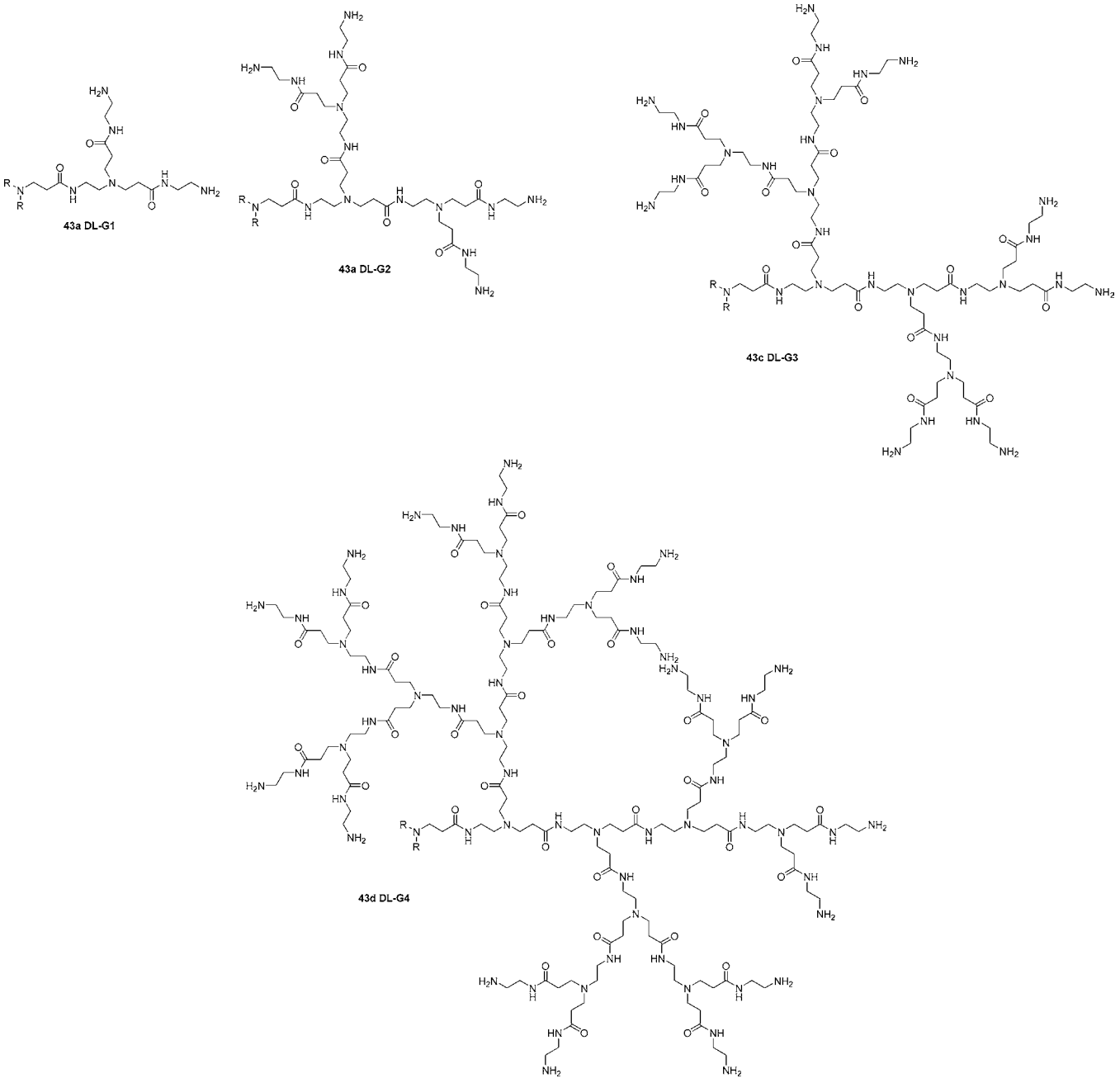

Figure 17. Chemical structures of dendritic-shaped polyamine lipids.

headgroup of the lipid can improve transfection efficiency and reduce toxicity, and would be worth exploring further as an *in vivo* delivery system for gene therapy.[133,134,141−143] Sen and Chaudhuri[133] described the design, synthesis, and gene transfection of cationic lipids bearing a monoguanidinium headgroup and an amine headgroup. Compared with cationic guanidinylated lipids (**50b**) containing the additional tertiary amine centers, lipids (**50c**) having the additional quaternized centers were observed to be remarkably transfection efficient. The results indirectly imply that the presence of an additional free tertiary amine center (with lower pK_a values) in the molecular structures of guanidinylated cationic lipids is perhaps not needed for noncholesterol based guanidinylated cationic lipids. In order to improve siRNA delivery efficacy, Santel et al.[142,143] synthesized a new cationic lipid (**51a**) including a monoguanidinium headgroup and two additional amine centers, which allowed for more efficient siRNA-binding as compared to other commercially available cationic lipids such as DOTAP or DOTMA. Similarly, Huang et al.[134] developed a novel nonglycerol based cationic lipid DSGLA (**51b**) containing both monoguanidinium group and amine centers, which synergistically enhanced the activity of siRNA as a therapeutic agent. Recently, Yingyongnarongkul et al.[34,131] reported the use of a solid-phase synthesis approach to generate several libraries of guanidinium-based cationic lipids (**52a−f**). The results revealed that 3β-[*N*-(*N*′-guanidinyl)-2′-aminoethyl)-*N*-(2-aminoethyl)carbamoyl] cholesterol exhibited the highest transfection efficiency with minimal toxicity among the guanidinium-based cationic lipids with primary amine. Furthermore, in contrast to cationic lipids (**52g** and **52h**) containing a monoguanidinium and secondary amine groups, cationic lipids (**53a**) with a bis-guanidinium and secondary

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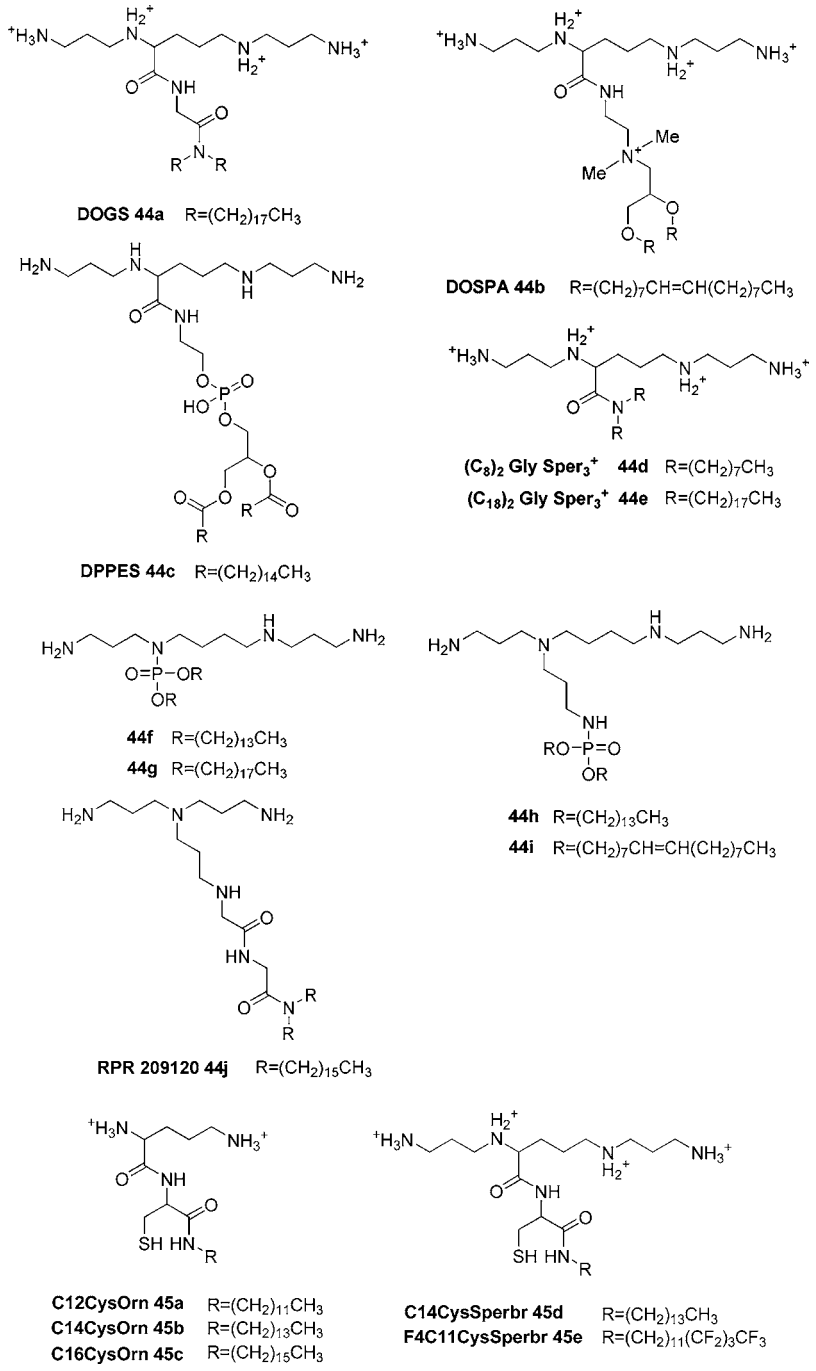

Figure 18. Chemical structures of T-shaped polyamine lipids bearing hydrocarbon chain.

amine groups generally had higher binding affinity and transfection efficiency.[34] Given the feasibility of a wide range of structural manipulations in the headgroup domains of cationic lipids, present findings are expected to further broaden the potential of cationic lipids with guanidinium headgroups for use in nonviral gene therapy.

The conjugate of bis-guanidinium and tertiary amine headgroup usually form a T-shaped headgroup. Lehn et al.[132,136,144−147] have been working at studying bis-guanidinium-based cationic lipids derivatives since 1996. Their earlier work focused on the synthesis and test of two bis-guanidinium cholesterol derivatives: BGTC (bis-guanidinium-trencholesterol, **54a**) and BGSC (bis-guanidiniumspermidine-cholesterol, **54b**).[132] Both BGTC/DOPE and BGSC/DOPE liposomes showed higher transfection efficiencies in a variety of mammalian cell lines when compared with Lipofectin (DOTMA/DOPE, 1/1, w/w). Additionally, BGTC (**54a**) was able to be successfully used for transfection when formulated without DOPE since it was found to be soluble in aqueous medium. However, further studies confirmed the particular ability of BGTC (**54a**) to efficiently transfect airway epithelial cells *in vivo* when it was used in formulations with DOPE.[136] In order to further explore the utility of guanidinium-based lipids, they synthesized and evaluated the transfection activity of the cationic lipid BGDA (**54c**, see Figure 21) containing a chain with a diacetylene unit and a bisguanidinium headgroup identical to that of BGTC (**54a**). Although it was efficient for *in vitro* transfection when formulated as cationic liposomes with

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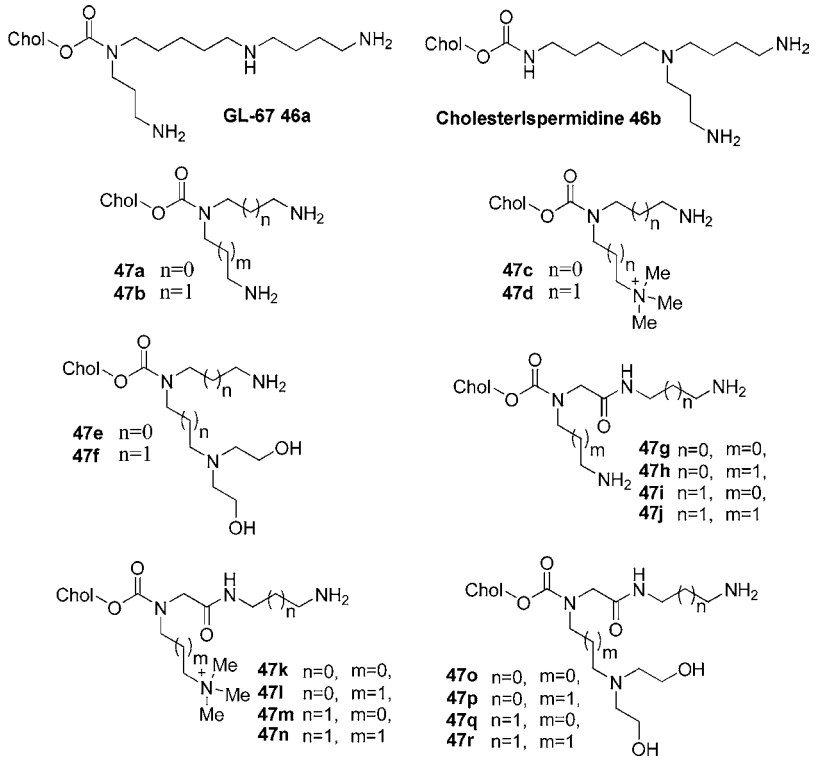

Figure 19. Chemical structures of T-shaped polyamine lipids bearing cholesterol.

a similar neutral diacetylene colipid (hydroxyethylenediacetylene, HEDA) and DOPE, BGDA/DOPE liposomes showed higher transfection efficiency in several mammalian cells, perhaps because the inclusion of DOPE increases the stability of the lipoplexes and has a strong tendency to form an inverted hexagonal phase, which acts as a fusogenic lipid promoting fusion of the lipoplexes with the endosomal membranes and subsequent release of the DNA into the cytoplasm.[145] To further evaluate the potentiality of the class of cationic lipids, they also reported the synthesis and characterization of four cationic steroid derivatives (**55a–d**) incorporating an acid-sensitive acylhydrazone linkage connecting a bis-guanidinium headgroup to either an unsaturated cholest-4-enone or a saturated cholestanone hydrophobic domain. BGBH-cholest-4-enone (**55b**) displayed relatively low cellular toxicity level and high transfection efficiency *in vitro* and further was capable of transfecting mouse airways *in vivo* when formulated as liposomes with DOPE as compared with BGTH-cholest-4-enone (**55d**), which might be related to differences in the length of the spacer and/or in the number of cationic groups per molecule.[146] As above discussions in polyamine–steroid conjugates, the transfection efficiency of lipids decreased with the increase in both length and total charge of headgroups, which was considered to be due to an increased flexibility of the headgroup of the longer compounds resulting in a more folded, rather than stretched, structure.[148]

Recently, Metwally and Blagbrough[149] described the synthesis of four novel spermine-derived fatty acid amide guanidines (**56a–d**), which were good candidates for nonviral delivery of siRNA to HeLa cells using self-assembled lipoplexes.

In order to study the impact of chemical modifications of guanidinium-based cationic lipids on their gene delivery properties, guanidine derivatives (e.g., cyclic guanidines (**57a**) and amidines (**57b** and **57c**)) were introduced into the headgroup domains of cationic lipids.[137,138,150–152] A known example of an amidine-containing lipid is diC_{14}-amidine (**57b**), which forms stable liposomes under physiological pH and temperature, and therefore it has been used, as other cationic lipids, to deliver nucleic acids and proteins into cells, with a certain efficiency.[138,150] Additionally, diC_{14}-amidine liposomes presented some interesting properties that are not shared by other cationic lipids, such as fusogenicity and immunomodulatority, and substantially inhibit TNF-α synthesis's behavior. Consequently, Elouahabi and colleagues[151] reported the use of the properties (the ability of free diC14-amidine liposomes to inhibit substantially TNF-α synthesis) to improve *in vivo* transfection efficiency of diC_{14}-amidine liposomes by pre-injection. Another example of amidine-containing lipid is TRX (**57c**, see Figure 22), showing structural transition from a cylinder to inverted cylinder upon mixing with pDNA and showing excellent transfection ability as well as less cytotoxicity.[152]

In sum, these results highlight the point that cationic lipids with headgroups composed of guanidinium functions represent an attractive option for gene delivery, perhaps because they have the following favorable characteristics: (a) the guanidinium group remains protonated over a much wider range of pH involved in liposome preparation and transfection than other basic groups due to its remarkably high pK_a values (13.5); (b) it can form characteristic pairs of strong parallel hydrogen bonds $N{-}H^{+}\cdots O^{-}$ with the phosphate anions of DNA; (c) it can also interact with nucleic acids bases via hydrogen bonds, especially guanine; (d) it is a component of the arginyl residues playing a key role in DNA-binding proteins such as histones and protamines.[135]

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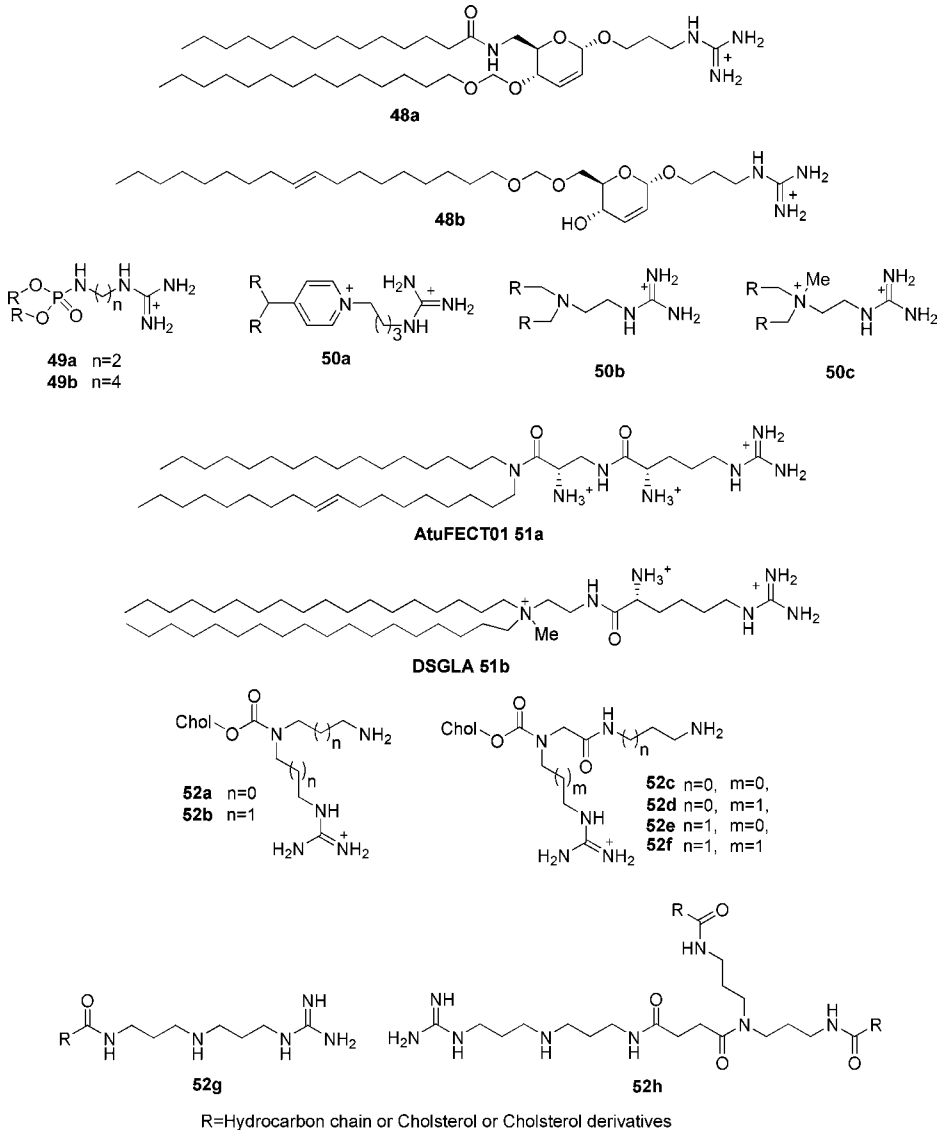

Figure 20. Chemical structures of cationic lipids bearing monoguanidinium headgroup.

EVOLUTION OF HETEROCYCLIC POLAR HEADS WITH DELOCALIZATION OF THE POSITIVE CHARGE

Cationic amphiphile with a pyridine heterocyclic ring headgroup was shown to have antiseptic and antibiotic properties in the 1940s. Since then, heterocyclic cationic amphiphiles have been widely researched. Several studies suggested that the type of heterocyclic headgroups affected the transfection efficiency of a given lipid.[153−155] Among the heterocyclic headgroups, pyridine, pyridinium, and their derivatives are by far the most frequently encountered and in particular proved to be very efficient, reaching or surpassing the transfection efficiency of commercial cationic lipid formulations, both *in vitro* and *in vivo*.[141,156−160]

Engberts and colleagues[141,155−158] have developed a series of pyridinium cationic lipids, called SAINT (synthetic amphiphile interaction), comprising cationic lipids and their congeners (**58a−f**, see Figure 23), and some of them have been reported to reach or surpass the performance of commercially available transfection reagents both *in vitro* and *in vivo*. The relative orientation of the pyridinium ring has a considerable effect on the transfection efficiency (meta (**59b**) > para (**59c**)). Additionally, the introduction of the additional groups (such as quaternary ammonium salt (**58g−i**) and guanidinium (**50a**)) in the delocalized monopyridinium headgroup of lipids can decrease the efficiency of transfection, probably due to difficulties related to genetic material unwrapping from the lipoplex.[141,156,157] However, cationic lipids **59a−e** can get better transfection effect when a biodegradable ester linkage was introduced. It was initially envisaged that the introduction of ester linkage reduced the stability of the lipoplexes through acid-induced hydrolysis of the lipids, facilitating the escape of DNA, which could also enhance intracellular metabolism, thus avoiding long-term persistence of the unnatural cationic lipids in cellular membranes and corresponding undesirable consequences for cellular functions. However, through the research of diester-linked lipids, they found that the esters appeared to hydrolyze by base catalysis, producing fast hydrolysis at physiological pH and higher stability in the endosomal pH range. Thereby, they thought the transfection potential and cytotoxicity were determined on cells in culture.[161] Other linkages such as ether, amide, urethane, or phosphonate ester groups have also been employed in pyridinium cationic

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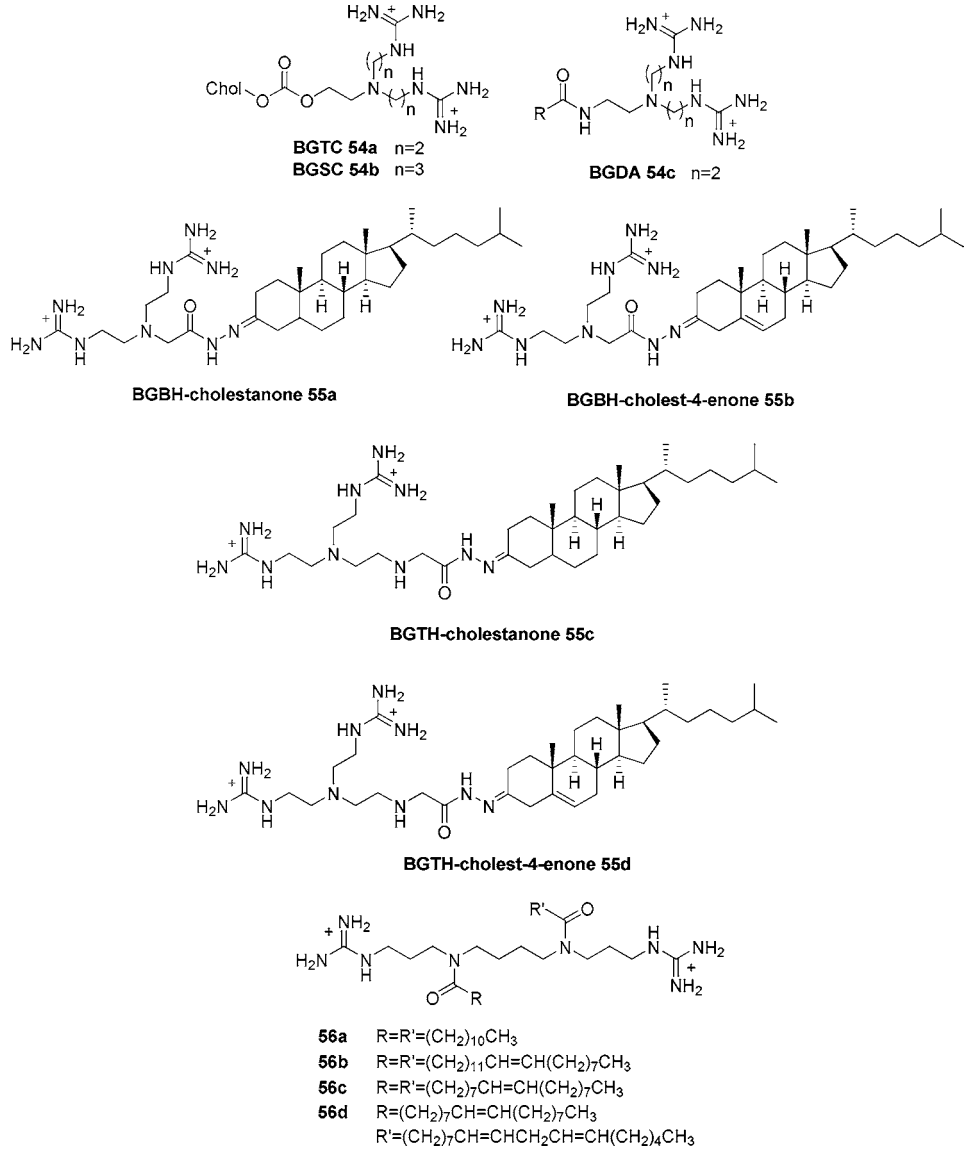

Figure 21. Chemical structures of cationic lipids bearing bis-guanidinium headgroup.

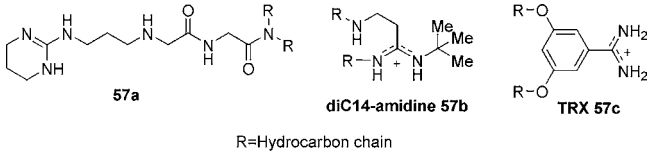

Figure 22. Chemical structures of cationic lipids bearing guanidine derivatives.

lipids.[162−165] The ether-linked lipids are more cytotoxic than the ester or urethane lipids, and this is possibly related to the fact that ether linker is too stable to be hydrolyzed in an acidic endosomal environment.[163]

To further research the structure−activity relationships of pyridinium cationic lipids, Engberts et al.[166] synthesized a new class of cationic lipids (Sunfish lipids **60a**). They found that the highest transfection efficiency was obtained with those lipids that are easily hydrated from fluid aggregates, and undergo a transition to the inverted hexagonal phase in the presence of plasmid DNA (p-DNA) at physiological ionic strength.

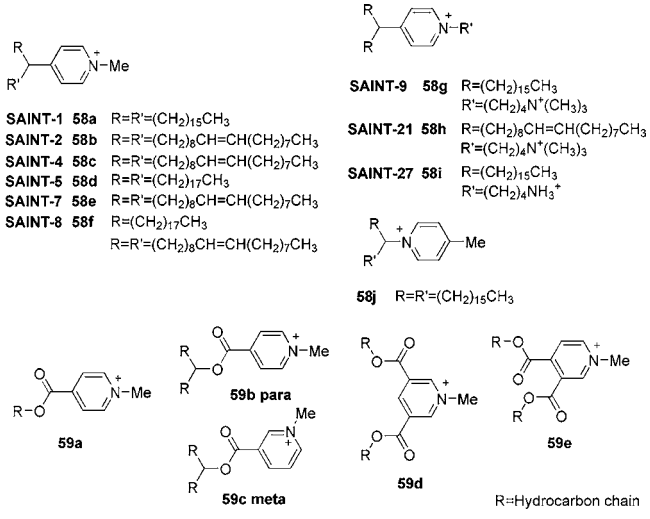

Figure 23. Chemical structures of cationic lipids bearing a pyridine heterocyclic ring headgroup.

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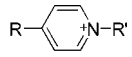

60a

R, R'=Hydrocarbon chain

Figure 24. Chemical structures of Sunfish lipids.

Another research study of pyridinium cationic lipids is from Balaban, Ilies, and colleagues,[167−171] who proposed a new strategy to access three series of collidinium cationic lipids (**61b−d**) via the reaction of pyrylium salts with primary amines. They found that the most efficient representative was the *N*-(1,3-dimyristoyloxypropane-2-yl)collidinium derivative **61c**, whose truncated cone shape can induce a higher radius of curvature of the lipid bilayer, thus generating smaller, more transfection-efficient liposomes. In order to study the structure−activity relationship of pyridinium cationic lipids, several new classes of trisubstituted pyridinium cationic lipids were synthesized via the same synthetic strategy (Figure 25).[159,172] The pyridinium lipid with amide linker (**61i**) or a

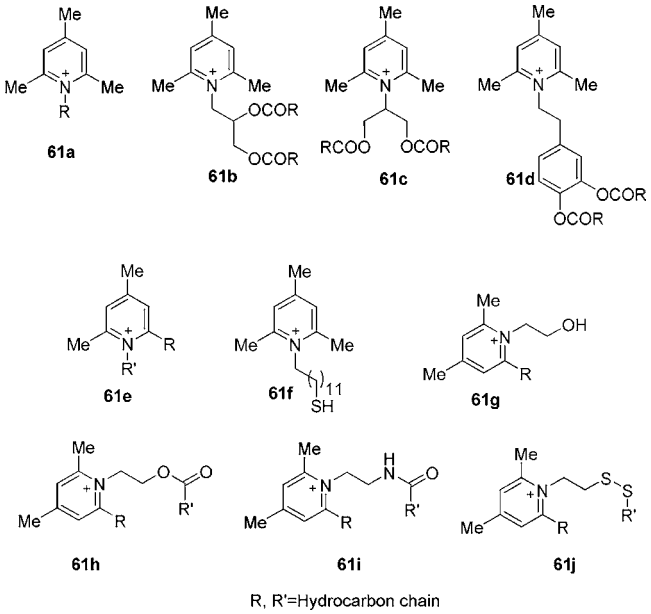

Figure 25. Chemical structures of trisubstituted pyridinium cationic lipids.

trans-configuration of the double bond in the fatty acid chain showed significantly higher transfection efficiency compared to its ester counterpart (**61h**) or counterpart with cis-configuration at the same fatty acid chain length, respectively. In addition, pyridinium lipids with a hydrophobic chain length of 16 displayed significantly higher transfection efficiency and lower cytotoxicity compared to their counterparts with other lengths.

Some research results showed that Gemini lipids generally exhibitd a transfection efficiency similar to or higher than that of their corresponding lipid analogues.[141,156] The spacer distance between the Gemini pyridinium headgroup has an impact on stability, DNA binding capability, cytotoxicity, and transfection efficiency (**62a−n**, see Figure 26).[141,159,160,173,174] Engberts et al.[141,156,159] revealed that cationic lipid (**62i**) with C_4 spacing obtained the highest transfection efficiency when compared with its counterparts containing shorter (C_3, **62m**) or longer (C_5, **62n**) spacers. Furthermore, in the case of the Gemini pyridinium series **63a**, the transfection efficiency increased monotonically with the spacer length when the spacer was elongated from five to eight carbon atoms, but the transfection efficiency of Gemini lipids with C_8 spacing was lower than that of its congener with C_2 spacing.

The introduction of labile bridging bonds in the structure of the spacers, such as ether bonds (**63b**) or disulfide linkage (**64a**,**b**), has a beneficial effect on transfection and cytotoxicity (see Figure 26).[159,160,174] For example, Gemini pyridinium lipid **63b** with an ether bond in the structure of the spacers was more efficient than its pentamethylene congener **63a**.[159] However, transfection activities of cationic lipids were diminished when pro-cationic amino moieties were introduced into the structure of the spacers (compounds **63c** and **63e**). It was presumably due to a very strong association of the lipids with the DNA that slows down the release of the plasmid *in vivo*, as proved by the Boc-precursors **63d** and **63f** showing 5−10 times more activity than their deprotected polycationic congeners **63c** and **63e**. Thus, to test the effect of substituting the Boc groups in **63f** with more lipophilic moieties, they were introduced either by alkylation or by acylation of **63e**. These liposomes were most active when coformulated with DOPE in a 1:1 molar ratio.

Hyvönen and co-workers[175,176] designed and synthesized two series of single-charged and double-charged 1,4-dihydropyridine lipids (**65a−i**) as transfection agents. Some of these compounds displayed relatively high transfection efficiency *in vitro*, in particular, the double-charged cationic amphiphiles (see Figure 27), which reveal some important structure−activity relationships. The double-charged derivatives (**65e−i**) with two methylpyridinium moieties in positions 2 and 6 with respect to the dihydropyridine ring have lower basicity at the dihydropyridine nitrogen to pK_a in the range 6−8; thus, they have buffering capacity at the endosomal pH range of 6.0−7.4, but the single-charged compounds with one pyridinium substituent in position 4 of the dihydropyridine ring does not have buffering capacity and is a weak transfectant. Additionally, the double-charged derivatives also abolish the buffering and the transfection activity when this crucial nitrogen in the dihydropyridine ring was methylated. Thus, the charge number of substituents and the buffering ability of the dihydropyridine ring nitrogen are important features of this structure and may contribute to the transfection efficacy.

Imidazole, imidazolium, and their derivative as headgroups of cationic lipids are becoming the focus of study among the heterocyclic groups. Three imidazole cationic lipids (**66a−c**) reported by Budker et al.[177] did not exhibit permanent cationic charges at the physiological pH, but they were able to bind to DNA and to encapsulate it after imidazole protonation at acidic pH, and showed good transfection properties. To increase the ability of imidazole cationic lipids to bind DNA, a permanent cationic function (at physiological pH) has been incorporated.[163,178,179] Mével and co-workers[93,180] have synthesized two lipophosphoramidates **66f** and **66g** bearing the *N*-methylimidazolium headgroup. Surprisingly, lipid **66f** can be prepared into liposomes with the lipophosphorimidate **66d** or with DOPE and proved to be very efficient for *in vitro* transfection; however, lipid **66g** was not able to be prepared into liposomes either in the absence or in the presence of DOPE. Presumably, this is caused by the spacer's length and/or the type of counteranion. Furthermore, they found that imidazolium lipophosphoramidate **66f**/DOPE lipoplexes gave the most efficient transfection with low toxicity (15%) compared with other headgroups, such as arginine methyl ester, lysine methyl

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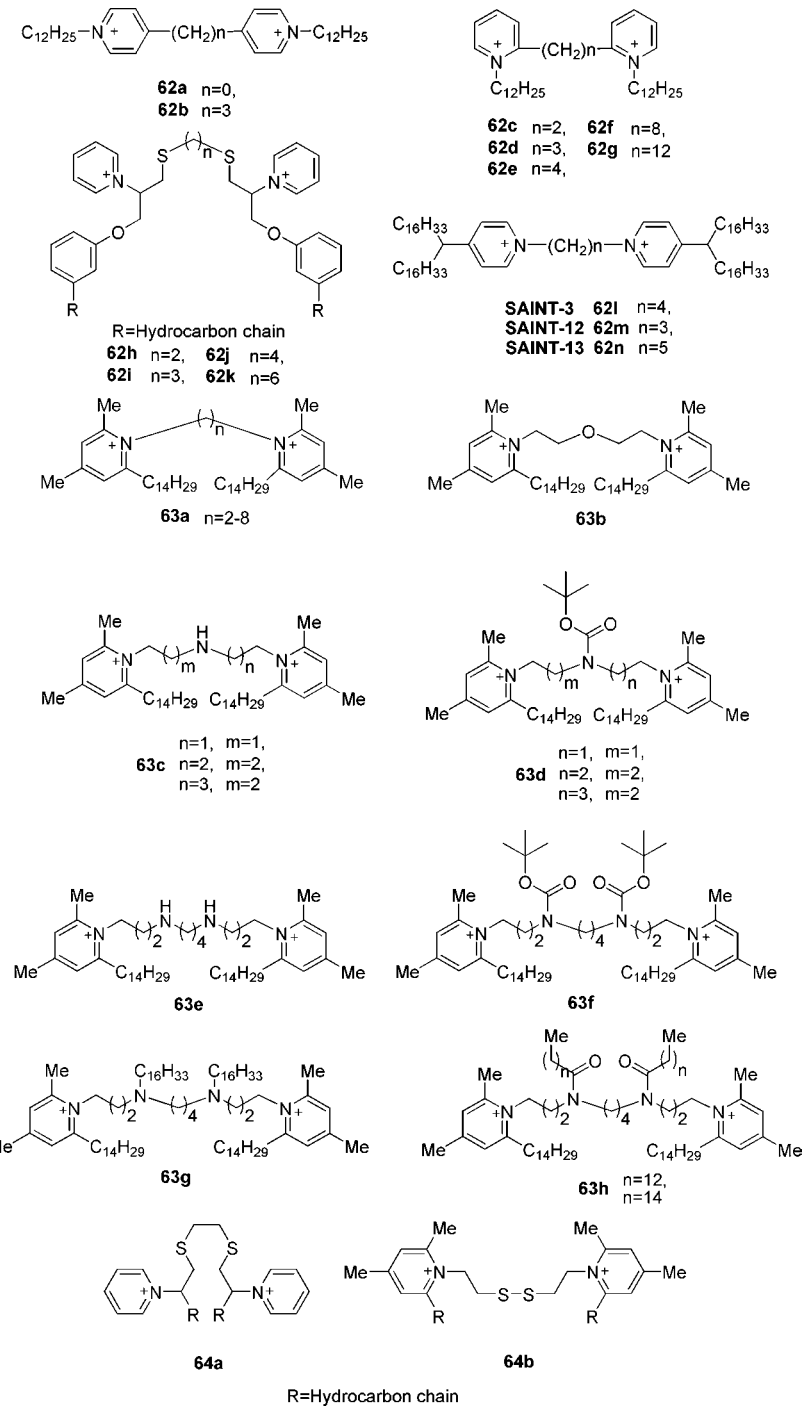

Figure 26. Chemical structures of Gemini pyridinium lipids.

ester, homoarginine methyl ester, ethylenediamine, diaminopropane, and guanidinium.

To further increase the cationic charge of the polar head and the ability of imidazolylated lipids to bind and compact DNA, two or more permanent cationic charges have also been introduced by the incorporation of other headgroups, such as another imidazolium (**67a–g**)[160,181] or tetraazacyclododecane (**68a–f**).[182,183] Six cationic lipids **68a–f** (see Figure 29) characterized by headgroups composed of imidazolium and tetraazacyclododecane functions have been designed and synthesized by Huang et al.[182−184] They found that transfection efficiencies of some of the cationic lipids could be dramatically increased in the presence of calcium ion (Ca^{2+}).

Other heterocyclic compounds used as hydrophilic moieties for cationic lipids include imidazolinium,[185] pyrrolidine,[80,178,186−188] piperazine,[189] cyclic polyamine,[189] tetraazacyclododecane,[182−184,190] morpholine,[163,178] chloroquine analogues,[191] nucleotides,[192] hoechst,[193] and so on.[165,179,194] In addition, Engel et al.[72] found that the ditertiary amine 1,4-diazabicyclo[2.2.2]octane (DABCO) constitutes a most useful species for the construction of an intriguing series of heterocyclic cationic lipids and synthesis of several series of

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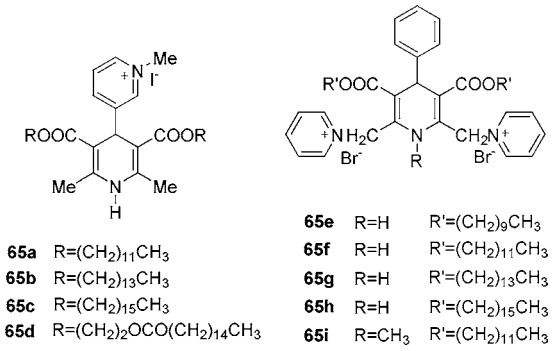

Figure 27. Chemical structures of cationic 1,4-dihydropyridine-based lipids.

symmetrical compounds that serve as cationic lipids or precursors thereof.

Solodin and co-workers[185] first reported the utilization of imidazolinium cationic lipids, DOTIM (**69a**), 1-[2-(hexadecanoyloxy)ethyl]-2-pentadecyl-3-(2-hydroxyethyl)-imidazolinium chloride (DPTIM, **69b**), and 1-[2-(tetradecanoyloxy)ethyl]-2-tridecyl-3-(2-hydroxyethyl) imidazolinium chloride (DMTIM, **69c**), as a synthetic carrier to deliver genes into cells. The hydroxyethylated DOTIM (**69a**) was the most effective among the three compounds both for *in vitro* transfection and for *in vivo* gene delivery.

Majeti et al.[186,187] demonstrated that a new panel of heterocyclic cationic lipids (**70a–c**) using pyrrolidinium with −OH, −NHBOC, or $NH_3^+Cl^-$ as hydrophilic headgroups was synthesized for gene delivery. They found that the substitution of hydroxyl functionalities in the headgroup domain of cationic amphiphiles did not necessarily enhance the *in vitro* gene transfer efficacies of cationic amphiphiles, and the higher gene transfer efficacies of lipids can be observed for the amino functionalities in the headgroup domain.

Recently, Islam and colleagues[189] synthesized a series of cationic lipids (**71a–r**) bearing substituted piperazine/cyclic polyamine (cyclen) derivatives, which can be conveniently formulated in lipoplex nonviral particles. Their observations indicated that lipid **71c** was good at condensation of plasmid DNA, while lipid **71d** was found to be poor related to condensation, but it was capable of delivering siRNA successfully. Cationic lipid **71d** includes two cholesteryl moieties, which may favor controlled interaction of the smaller siRNAs to form a lipoplex that is capable of efficient delivery of the siRNAs to their cytoplasmic site of action.

Since some tetraazacyclododecane-based cationic lipids showed better transfection efficiency than those with other lipophilic groups in recent investigation.[182−184] Liu et al.[190] designed and synthesized three novel amphiphilic lipids (**72a–c**; Figure 33) containing the structures in which protonated cyclen was linked to different hydrophobic groups via a PNA (peptide nuclear acid)monomer linkage. **72c**/DOPE liposome gave better transfection efficiency compared with two other lipids **72a** and **72b** in association with DOPE and Lipofectamine 2000, indicating that the tetraazacyclododecane-based cationic lipids have great potential to be efficient nonviral gene vectors.

Some comparative studies on transfection efficiency of lipids with different headgroups have been reported.[163,178,190,194] Gao and Hui[194] have described the transfection efficiency of a series of cationic lipids with different headgroups, including linear amine (**73b**), imidazole (**73c**), morpholine (**73d**), pyridine (**73f**), polyamine (**73g** and **73h**), and piperazine (**73a**, **73e**, **73i**, and **73j**), and observed that cationic lipids containing heterocycles as the headgroup gave better efficiency of gene transfer in comparison with lipids having linear primary amines or polyamines as the headgroup. Within the group of cationic lipids containing heterocycles, lipids with piperazine (**73a**, **73e**, **73i**, and **73j**) and morpholine (**60d**) could mediate more efficient gene delivery *in vitro*. However, **73i** bearing a piperazine group was relatively less active, it might be due to the fact that **73i** contains dual cholesteryl moieties, whose overall structure might be too bulky to interact with DNA. But the authors thought that the dual cholesteryl groups within the lipoplex may favor controlled interaction of the smaller siRNAs to form a lipoplex that was capable of efficient delivery of the siRNAs to their cytoplasmic site of action as the discussion above;[189] thus, **73i** may be a good transfection agent for the delivery of the siRNAs.

Although a different concept of DNA−cationic lipid interaction was tested using Hoechst 33258, which intercalates into DNA rather than binding electrostatically,[193] many researchers generally allow interactions between the cationic lipids, the negatively charged DNA, as well as cell membrane, through charge/charge interactions. Thereby, some cationic lipids bearing positively charged heterocyclic rings have been incorporated in the comparative studies recently.[163,165,179] Bajaj and colleagues[165] have synthesized eight cholesterol-based cationic lipids (**74a–l**) differing in the headgroup, and found that **74f** containing a tertiary amine and one pyridinium group as headgroup showed the maximum transfection efficacy in the presence of serum. Cationic lipids (**74i–l**) were synthesized

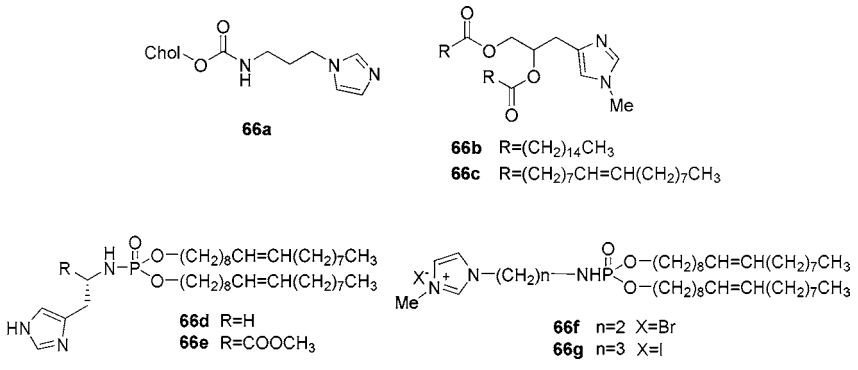

Figure 28. Chemical structures of cationic lipids with imidazole or imidazolium headgroups.

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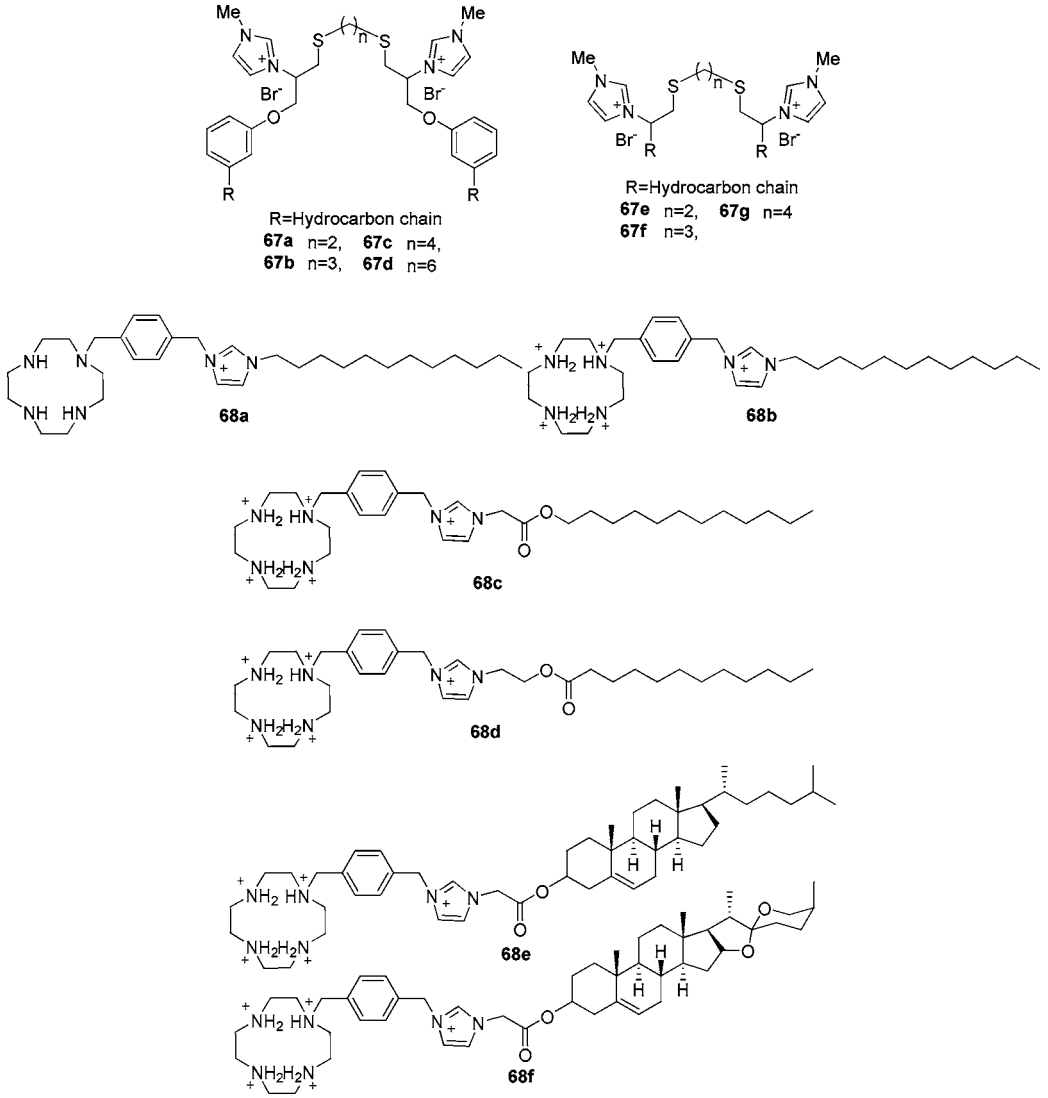

Figure 29. Chemical structures of imidazolium cationic lipids bearing other headgroups, such as another imidazolium, tetraazacyclododecane, or hydroxyethyl group.

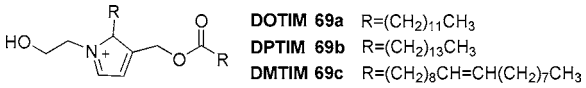

Figure 30. Chemical structures of imidazolinium cationic lipids.

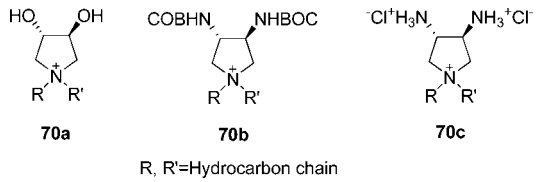

Figure 31. Chemical structures of heterocyclic cationic lipids using pyrrolidinium with −OH, −NHBOC, or $NH_3^+Cl^-$ as hydrophilic headgroups.

following the same synthetic procedure, and formed complexes by loading the single-walled CNTs (carbon nanotubes). These complexes containing CNTs displayed higher DNA binding, nearly comparable release, and greater stability and compatibility in FBS (fetal bovine serum) compared to complexes which did not contain any CNTs.[195] In addition, formulations (**75e**) that contained GR (disperse exfoliated graphene) and/or solubilize tamoxifen citrate (TmC) were found to be particularly efficient agents in delivering the drug to the cells, compared to the suspensions devoid of GR against a number of transformed cell lines.[196]

To optimize the structure of cationic lipids in order to decrease the toxicity and increase gene transfer activity, Medvedeva et al.[163] synthesized and studied new biodegradable cationic lipids (**75a−f**) based on the cholesterol derivatives with positively charged pyridine, methyl imidazole, or *N*-methylmorpholine headgroup connected by an ester, ether, or carbamate linker. Results showed that the cholesterol-based cationic lipids containing positively charged pyridine and methyl imidazole headgroups with either ester or carbamate linkers (**75a**, **75c**, and **75d**) exhibited lower toxicity and higher transfection activity as compared to the cationic lipids with the *N*-methylmorpholine head groups and/or the ether linker (**75b**, **75e**, and **75f**). They thought that the size of the complexes was a major factor to influence their transfection activity. Then, Maslov and co-workers[179] prepared a number of cholesterol-based cationic lipids (**76a−j**) differing in the position of the positively charged group (pyridinium, *N*-methylimidazolium,

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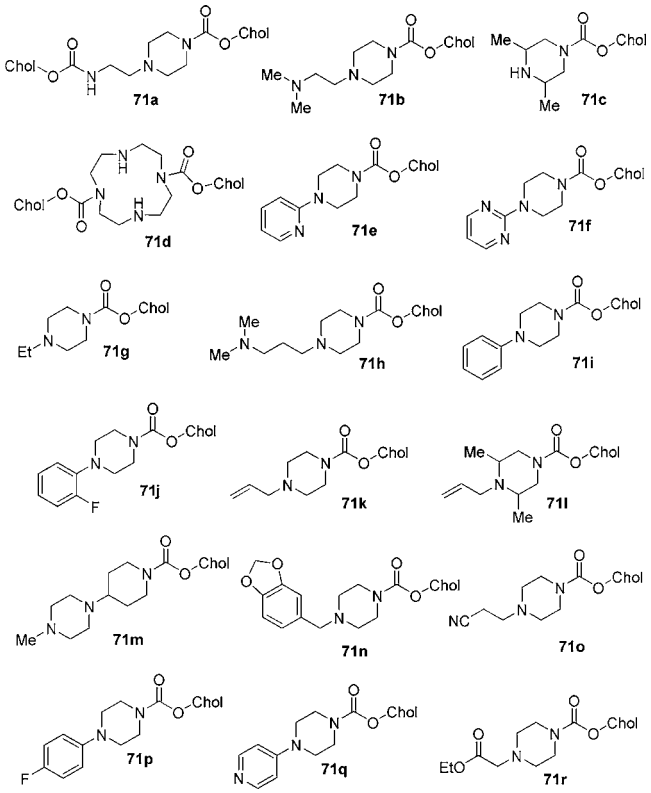

Figure 32. Chemical structures of cationic lipids bearing substituted piperazine/cyclic polyamine (cyclen) derivatives.

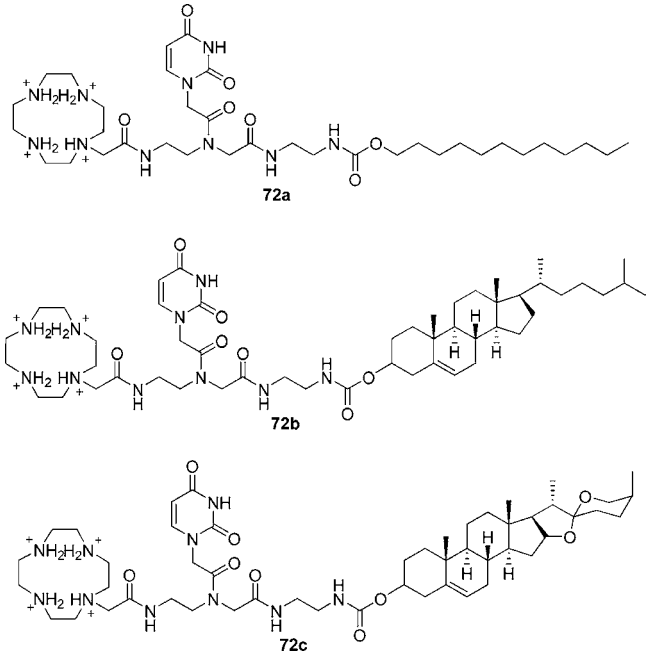

Figure 33. Chemical structures of tetraazacyclododecane-based cationic lipids.

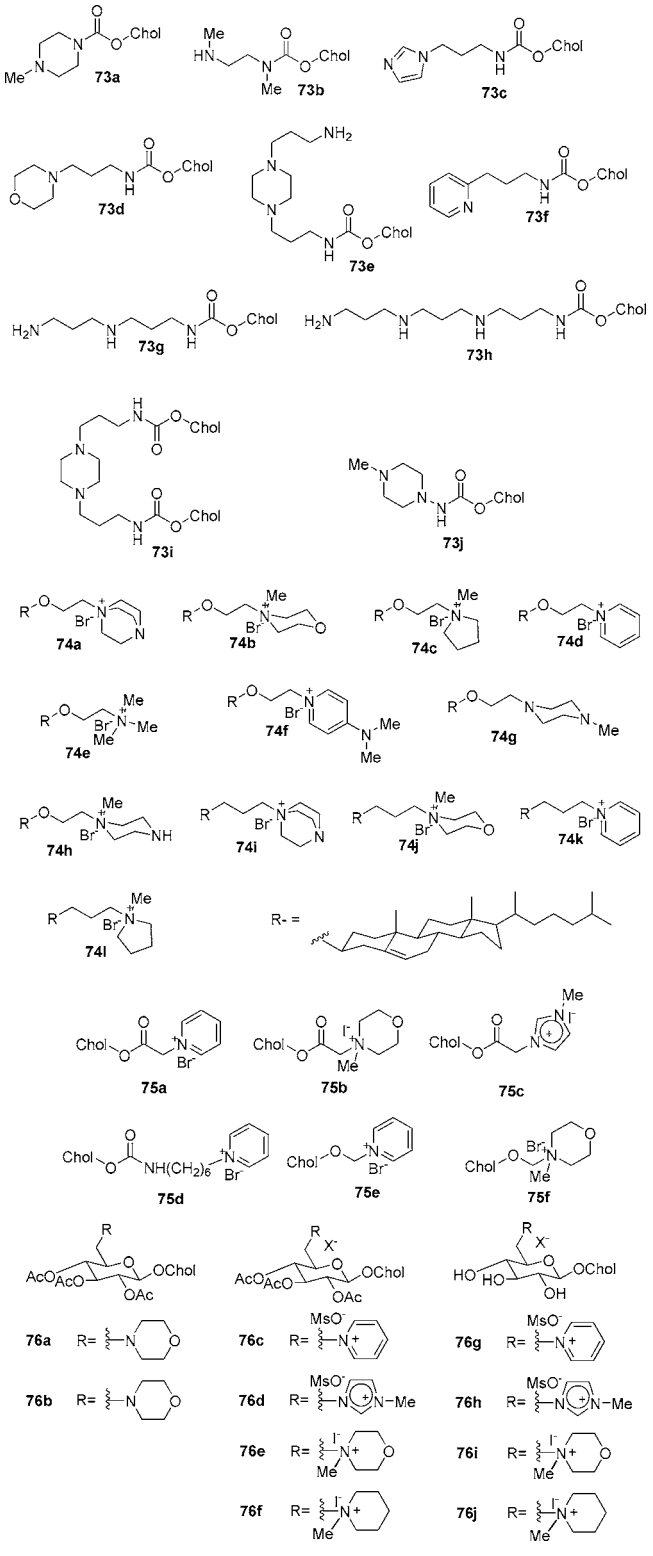

Figure 34. Chemical structures of cationic lipids with different headgroups include linear amine, polyamine, and heterocyclic ring headgroups.

N-methylmorpholinium, and *N*-methylpiperidinium) and the type of linker between the carbohydrate and the glycerol fragment. They found that lipoplexes formed by the cationic lipid (**76j**) with an *N*-methylpiperidinium headgroup displayed the most pronounced down-regulation of EGFP expression both in the presence and in the absence of serum (up to 30%).

As mentioned above, heterocyclic cationic lipids in general and pyridinium or imidazolium cationic lipids in particular displayed higher transfection efficiency and lower cytotoxicity both *in vitro* and *in vivo* compared with classical transfection systems. These attributes are believed to be due to the delocalization of the positive charge on several atoms of the

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heterocyclic polar head that confers a good balance between binding and releasing of the nucleic acid, which is essential for superior transfection efficiency.

RECENT ADVANCES WITH NOVEL HEADGROUPS BY INTRODUCING THE TARGETING AND BIOCOMPATIBILITY FUNCTIONALITIES

In order to obtain the relationship between hydrophilic headgroups and transfection efficiency, some special gene vectors have been designed and synthesized. Despite much progress, these novel vectors must overcome multiscale challenging requirements including biocompatibility, nonspecificity of targeting, stability in blood serum, and the ability to traverse biological barriers. An alternative strategy for nonviral vectors is direct chemical modification of nonviral vectors by introducing the moieties of the targeting and biocompatibility functions for gene delivery.

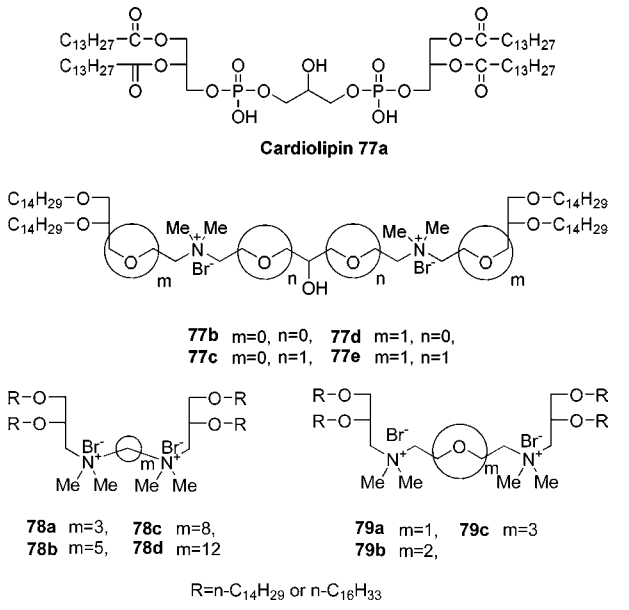

Figure 35. Chemical structures of Cardiolipin and cationic cardiolipin analogue (CCLA).

Cardiolipin (**77a**) is an important component of the inner mitochondrial membrane and has two negatively charged phosphate groups which were replaced with quaternary ammonium groups to provide cationic cardiolipin analogue (CCLA, **77b–e**). Some CCLA-based liposomes were tested for the DNA transfection efficiency and also used to determine the therapeutic efficacy of c-raf small interfering RNA (siRNA) in mice. Lipid **77c** was less toxic and has been shown to transfect different cell lines with up to 7-fold more efficiently compared to commonly used Lipofectin.[60,197] Bhattacharya et al.[198–200] synthesized a series of cardiolipin mimicking Gemini lipid analogues (**78a–79c**) upon variation of length and hydrophilicity of the space between the cationic ammonium headgroups and lipid hydrocarbon chain lengths. These cationic cardiolipin analogue formulations showed a significant enhancement in gene transfection activities as compared to that of lipofectin.

Nucleolipids (**80a–g**) that generally contain both nucleic acid recognition headgroups and lipophilic alkyl chain components have also attracted the researchers' interest because of their intrinsic molecular recognition and cell penetrating ability.[201–209] Barthélémy et al.[202] described the synthesis and preliminary physicochemical investigations of a purine nucleoamphiphile (**80c**) bearing two oleyl fatty acid chains. At the same time, they also described a new neutral DNA amphiphile (**80d**) for binding to nucleic acid and subsequent supramolecular assembly formation.[203,204] They found that the amphiphile possessed the capability of efficiently binding the nucleic acid double helixes and provide further motivation for the design and evaluation of new amphiphiles. In order to expand the repertoire of available functional nucleolipids, amphiphilic uridine analogue **80g** combining an oleic acid chain, a positively charged L-proline residue, and a polar oligoethylene glycol chain in its skeleton has been prepared and characterized by Simeone et al.[207] They expected that a relevant enhancement of the biological properties of cationic lipids related to **80g** can be presumably achieved by simply varying the functionalizing arms (cationic aminoacid, oligoethylene glycol, and lipid chains) using different natural and/or artificial analogues.

Another example is glycosylated cationic lipids,[210,211] some of whose headgroups can serve as markers for cellular recognition, such as mannose and galactose.[212–216] Hashida et al.[212–214] synthesized a series of cholesterol derivatives possessing the cationic charge necessary for DNA binding and mannose (**81a**) or galactose residues (**81b**) as targetable ligand for certain cells or organs as shown in Figure 33. Man-C4-Chol (**81a**) that has a positive charge and a mannose residue displayed high transfection activity in liver. It is possible that its mannose residues can be deposited on the liposome surface without adversely affecting the binding ability of cationic liposomes to DNA and can also be recognized by mannose receptors both *in vitro* and *in vivo*.[214] In addition, cationic liposome–DNA complexes containing Gal-C4-Chol (**81b**) having a galactose residue and a positive charge were efficiently recognized by asialoglycoprotein receptors, internalized, and led to gene expression in HepG2 cells.[215] In addition, it was also an efficient carrier of siRNA for hepatocyte-selective gene silencing following intravenous administration.[216] Recently, Yang et al.[217] designed and synthesized a cationic lipid (**82a**) bearing a galactose group and a carbamate-linked quaternary ammonium for gene delivery to hepatocytes.

In order to explore structure–activity relationship of glycosylated cationic lipids, two novel series of cationic glycolipids with cyclic (lipids **83a–e**) and open D-galactose heads (lipids **83f–k**) as well as varying spacer arm lengths in between the sugar and positively charged nitrogen atoms have been synthesized for gene delivery *in vitro* and *in vivo*. The results demonstrated that glycosylated cationic lipids with cyclic D-galactose head require longer spacer arms than their open D-galactose head counterparts for efficient gene transfection, and both series hold equal promise for selective gene targeting to liver under systemic settings.[218]

Glycosylated cationic lipids with two same acyclic sugar-heads are generally Gemini lipids and have been widely used in gene transfection.[59,219–222] Engberts et al.[59,219–222] synthesized a series of sugar-based Gemini lipids (**84a–l**) with open mannose and glucose heads containing varying spacer units in between two tertiary amine headgroups. They found that the effect of the headgroup, glucose vs mannose, or the effect of the spacer, C6-alkyl vs C4-alkyl and C6-alkyl vs ethylene oxide, did not remarkably appear to modulate the level of transfection to a significant extent. However, the spacer, C6-alkyl vs ethylene oxide, exerted a remarkable effect on toxicity, as lipids with

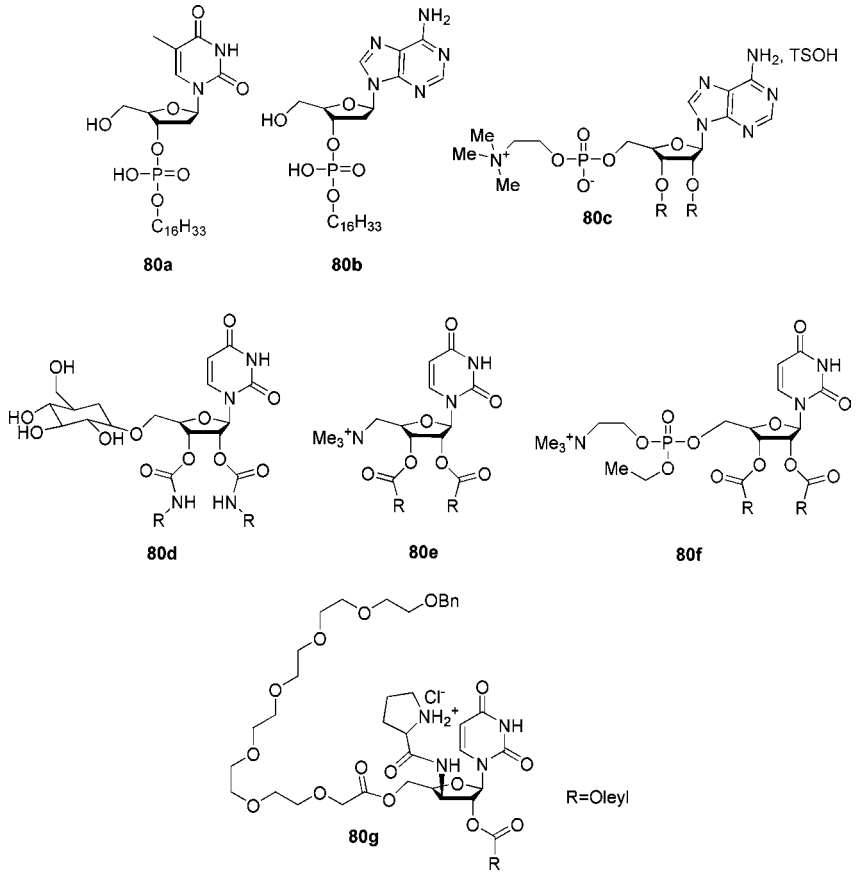

Figure 36. Chemical structures of nucleolipids and their analogues.

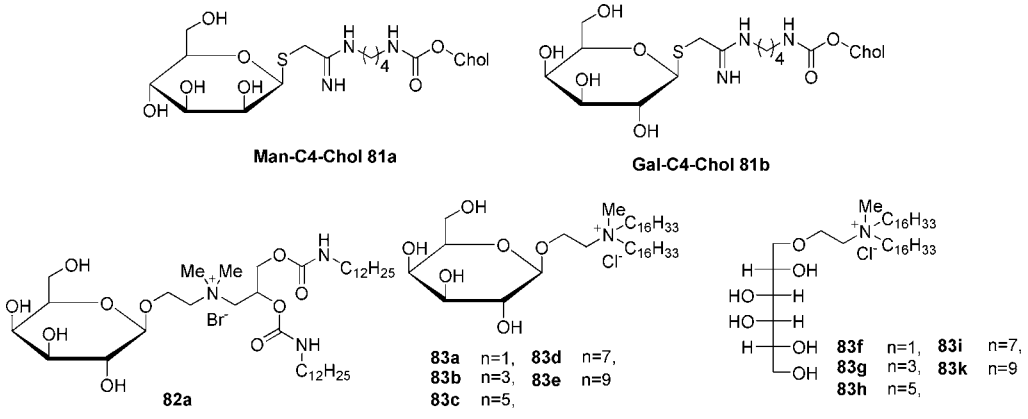

Figure 37. Chemical structures of glycosylated cationic lipids.

ethylene oxide displayed lower toxicity than those with C6-alkyl.

In addition to glycosylated cationic lipids with monosaccharide headgroups, lipids containing aminoglycoside cationic headgroups have exhibited significant gene transfer as compared to various commercially available agents (see Figure 39). Lehn and colleagues[223−226] designed and synthesized a series of aminoglycoside cationic lipids characterized by headgroups composed of an aminoglycoside or its guanidinylated derivative. KanaChol **85c**, an aminoglycoside lipid synthesized from konamycin, showed significant gene transfer both *in vitro* and *in vivo*; because of its three free amine groups, this molecule would bind to and condense DNA at physiological pH and the cholesterol subunit would in addition facilitate the cellular uptake of the lipoplexes generated. Shortly after the success of KanaChol **85c**, TGKC **85d**, the fully guanidinylated derivative of KanaChol **85c**, was synthesized and showed gene transfer capability. Recently, a series of aminoglycoside cationic lipids (**85e−h**) with a neamine headgroup, which incorporates rings I and II of the natural antibiotic aminoglycoside neomycin B, have been successfully used for siRNA delivery. The gene transfection experiments revealed interesting structure−activity relationships and allowed us to identify a formulation incorporating a small neamine derivative as a highly efficient gene delivery system.

Some novel cationic lipids that incorporate a paramagnetic contrast agent and/or a fluorophore onto a single molecular framework have been synthesized to obtain lipoplexes with

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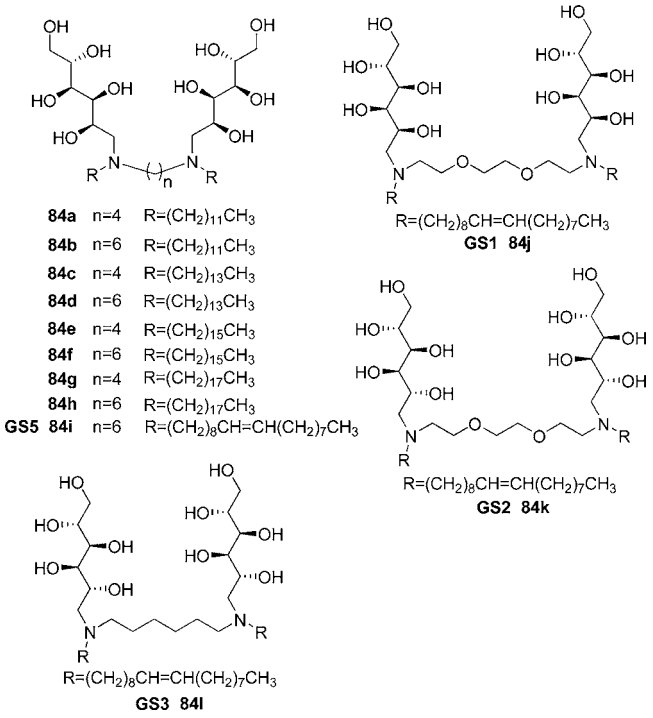

Figure 38. Chemical structures of sugar-based Gemini lipids.

magnetic resonance imaging (MRI) contrast properties. These paramagnetic contrast agents are most commonly gadolinium (Gd) or iron (Fe). Generally, gadolinium displays lower relative sensitivity compared with iron(III)-based contrast agents.[227−230] With the aim of following the biodistribution of lipoplexes and correlating their transfection ability with *in vivo* biodistribution, gadolinium-chelating cationic lipids (**86a**) have been synthesized to obtain lipoplexes with MRI contrast properties. Results showed that cationic lipid/DNA complexes displayed significant levels of transgene expression after *in vitro* transfection and can be observed *in vivo* using magnetic resonance imaging after intratumoral administration. However, it could induce a positive contrast change in the MRI as opposed to iron-induced darkening of images, which is often undesirable in biological systems.[231] Thereby, some new gadolinium-chelating cationic lipids (**86b−d**) have been synthesized to solve this problem (see Figure 40).[232−234] Jorgensen and Miller et al.[232−234] have synthesized a number of paramagnetic gadolinium-chelating lipids (**86b-d**) for liposomal cell labeling and visualization by MRI, with the additional incorporation of a separate fluorescent lipid in the liposome formulation or a fluorophore group in the lipids in order to create a robust bimodal liposome. They found that the bimodal lipidic molecule (Gd·DOTA·Rhoda·DSA **86d**) containing both fluorophore and contrast agent signatures on the same structure was more effective and sensitive than the single signature

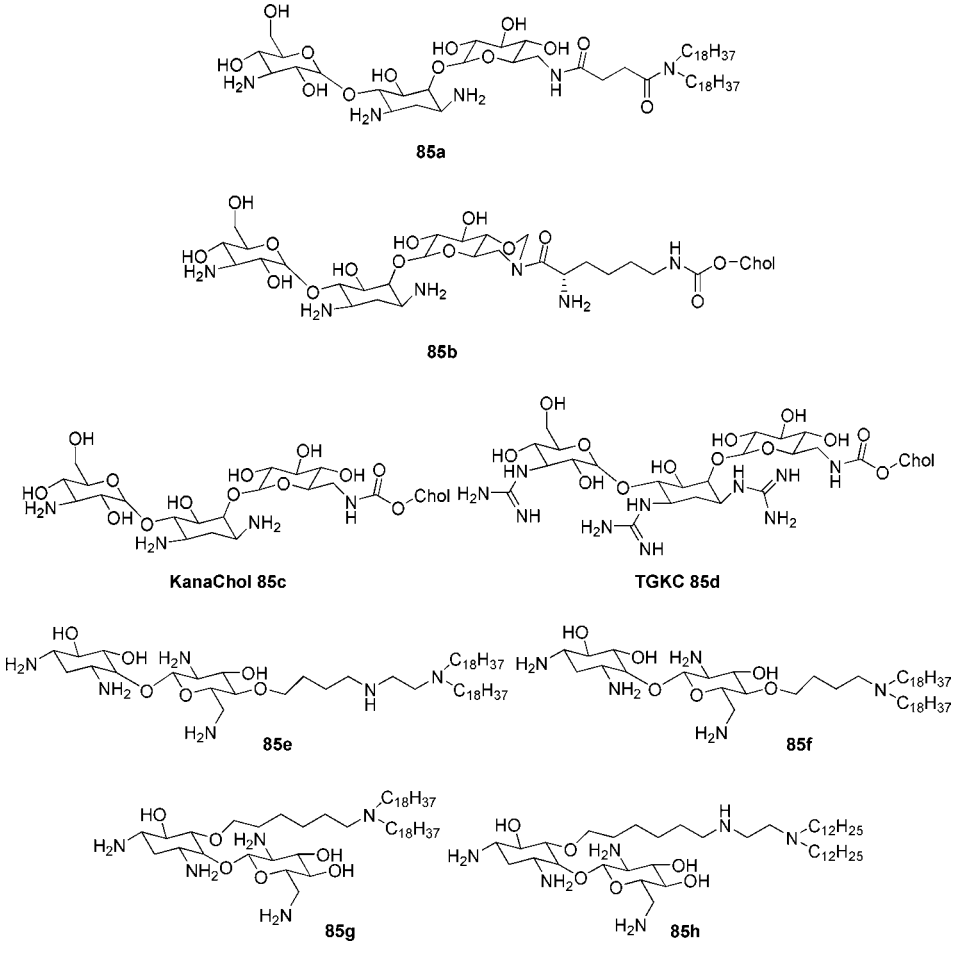

Figure 39. Chemical structures of aminoglycoside cationic lipids.

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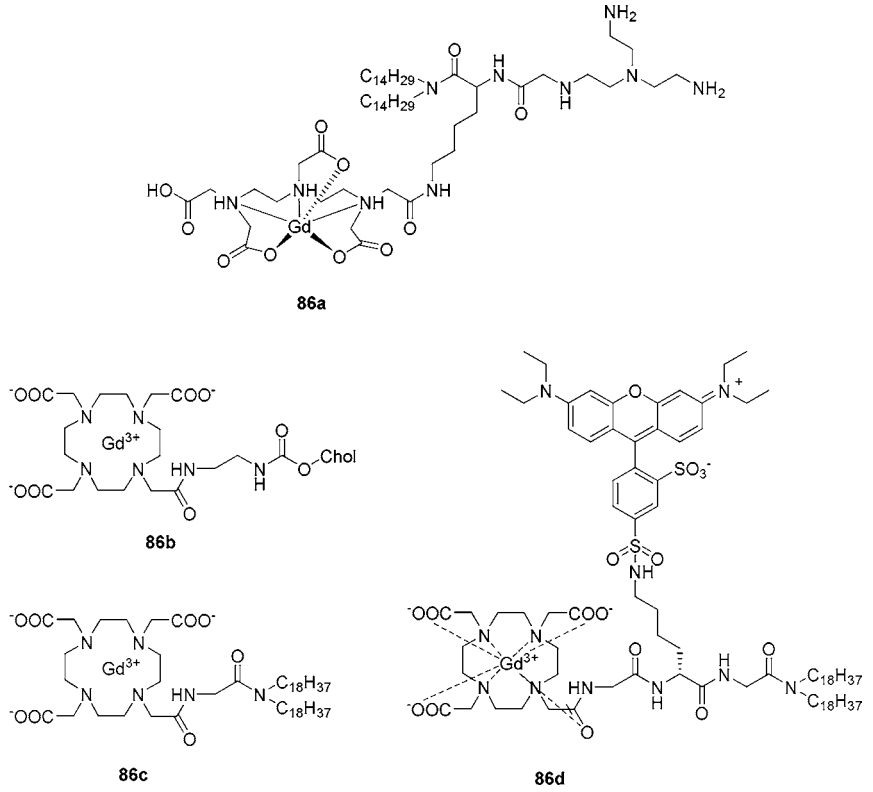

Figure 40. Chemical structures of gadolinium-chelating cationic lipids.

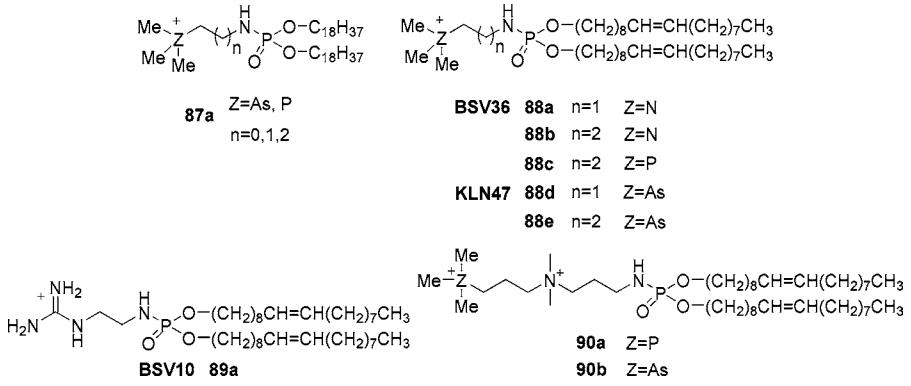

Figure 41. Chemical structures of cationic phosphonolipids and lipophosphoramidates.

paramagnetic lipid Gd·DOTA·DSA at cellular labeling and tumor MRI.[234]

In manipulating the cationic headgroup, replacing the nitrogen with phosphorus or arsenic afforded phosphonium and arsenium headgroups can also improve transfection efficiency and reduce toxicity.[57,235−243] Floch and co-workers[235,236] replaced the ammonium cation of phosphonolipids (**87a**) with either phosphonium or arsenium groups, and showed that phosphonolipids with arsonium and phosphonium headgroups exhibited significantly lower cytotoxicity than the ammonium analogues. Structure−activity relationships of these arsonium and phosphonium phosphonolipid derivatives showed that increasing the number of methylene units (n) between the phosphonate group and the cationic moiety ($n = 2 > n = 1 > n = 0$) can increase *in vitro* transfection efficiency in HeLa cells.[235] It has also been proved that phosphonolipid with an arsonium headgroup was the most efficient vector. Later, some results showed that the replacement of the phosphonate group by a phosphoramidate functional group was generally beneficial in terms of efficiency and cytotoxicity.[237,238] A trimethylarsonium lipophosphoramidate (KLN47 **88d**) was one of the most efficient vectors in this series, and it also displayed superior efficiencies over its analogues possessing either a trimethylammoniumpolar head (BSV36 **88a**) or a guanidinumpolar head (BSV10 **89a**) (see Figure 41).

These promising results encouraged some researchers to investigate the modification to phosphoramidate lipids, such as the insertion of a permanent dicationic charge, and to evaluate their influences on transfection efficiency.[57] Cationic phosphoramidate lipids containing an imidazolium group and a phosphonium or arsonium moiety have been efficient for *in vitro* transfection experiments, but they were less efficient than other dicationic lipophosphoramides without the imidazolium cationic group. However, compound **90a** (see Figure 41), whose polar head is characterized by the presence of a central ammonium and a terminal phosphonium, was the most

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efficient transfection reagent in A549 and HeLa cells. Interestingly, its cytotoxicity was about one-third lower than that of the monocationic vector at a similar charge ratio. Overall, these dicationic lipophosphoramidates gave a slightly better efficiency in comparison with their monocationic analogues for *in vitro* gene delivery.

For a number of gene therapy applications, the incorporation of some groups (such as mannose, galactose, or fluorescent groups) into the headgroup domain of cationic lipids has attracted great attention due to their potential in directing the therapeutic genes to the target cells, and it may help minimize adverse effects such as cytotoxicity or immune reactions, as well as maximizing the efficacy of the therapeutic response. Furthermore, cationic lipids such as cardiolipin or lipophosphoramide derivatives, whose chemical structures are similar to some of molecules in living organisms, show low toxicity level and high transfection efficiency and may provide an optimistic outlook for the development of lipid-based systems that are more applicable for human gene therapy.

■ CONCLUSION

In recent years, tremendous progress has been made in the design and synthesis of cationic lipids for gene delivery as well as the application of a battery of techniques from which structure−activity trends have emerged. However, solid conclusions are rarely obtained, particularly with regard to the relationships between the transfection activity of cationic lipid and structure feature of its headgroup. The hydrophilic headgroup which is positively charged is responsible for the interaction between liposomes and DNA, and between lipoplexes and cell membranes or other components of the cell. The different types of headgroups can be categorized as follows: quaternary ammoniums, amines, amino acids or peptides, guanidiniums, heterocyclic headgroups, and some unusual headgroups.

Some amine groups with different degrees of substitution are predominantly employed as the headgroups of cationic lipids. For quaternary ammonium headgroup, the hydroxyls or other quaternary ammonium groups are incorporated into cationic lipids to affect the complexation and the release of DNA, the membrane-forming properties, the surface hydration of the membranes formed from these lipids, to further improve the transfection efficiency. Additionally, cationic lipids containing primary, secondary, and/or tertiary amine headgroups improved transfection efficiency significantly by the introduction of another amine headgroup or the alteration of the spacer lengths. Cationic lipids including polyamine headgroups have pH-buffering functions, which are generally better at DNA binding than other amine counterparts. It is possible that their pH-buffering function inhibits the early endosomes from maturing and delays the fusion between endosomes and lysomes, thus increasing the stability of plasmid DNA in cells, since acidic lysomes are where most substances are destroyed. However, the cytotoxicity of these compounds stimulated investigation of alternative functionalities, including cleavable bonds and the shape, the spacing, and the number of amine functional groups.

Cationic lipids using guanidinium and its derivates as the cationic domain can also improve the efficiency of transfection. The highly basic guanidinium function remains protonated over a wide range of pHs involved in liposome preparation and transfection, leading to DNA binding that is less sensitive to pH variations. These groups can form characteristic pairs of parallel hydrogen bonds with the DNA phosphate anions, and can also interact with nucleic acids via hydrogen bonds, which highlight the point that cationic lipids bearing guanidinium functions represent an attractive option for gene delivery. Furthermore, heterocyclic cationic lipids (pyridinium or imidazolium cationic lipids in particular) generally exhibit higher transfection efficiency and lower cytotoxicity in comparison to classical transfection systems. These are believed to be due to the delocalization of the positive charge on several atoms of the heterocyclic polar head that confers a good balance between binding and releasing of the nucleic acid. Another trend of cationic lipid development is the incorporation of some groups such as mannose, galactose, or fluorescent groups into the headgroup domains of cationic lipids, which can improve transfection efficiency *in vitro* and *in vivo*. Besides all, some other cationic lipids (such as cardiolipin or lipophosphoramide derivatives) whose chemical structures are similar to those of some molecules in living organisms, display low cytotoxicity and high transfection efficiency. It may provide an optimistic outlook for the development of lipid-based systems that are more applicable for human gene therapy.

In sum, transfection efficiencies of cationic lipids could be affected by the gene being delivered, except for the structure of cationic lipids, whether it is *in vitro* or *in vivo*, and the type of biological response being measured. Biological studies have shown that pDNA is delivered to cells more efficiently than linear DNA, because pDNA is compacted with a large amount of counterions, yielding a lower effective negative charge compared to linear DNA, and therefore a lower amount of cationic lipid is needed.[244] Furthermore, when researchers design cationic lipids for gene delivery, some factors including the nature (such as the structure and shape of cationic headgroup) and density (such as the number and the spacing of cationic headgroup) of head functional groups should be taken into great consideration. It would be very important for scientists to have a good understanding about the effects of the different headgroups of cationic lipids on transfection efficiency and to help the rational design of novel cationic lipids with high transfection efficiency.

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Notes

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