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Functional lipids and lipoplexes for improved gene delivery

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Abstract

Cationic lipids are the most common non-viral vectors used in gene delivery with a few currently being investigated in clinical trials. However, like most other synthetic vectors, these vectors suffer from low transfection efficiencies. Among the various approaches to address this challenge, functional lipids (i.e., lipids responding to a stimuli) offer a myriad of opportunities for basic studies of nucleic acid–lipid interactions and for *in vitro* and *in vivo* delivery of nucleic acid for a specific biological/medical application. This manuscript reviews recent advances in pH, redox, and charge-reversal sensitive lipids.

Keywords

Lipid; Amphiphile; Lipoplex; Gene delivery; DNA; siRNA; Synthetic vector; Functional; Responsive

1. Introduction

Gene therapy is an approach to treat disease by either modifying the expression of a gene or correcting an abnormal gene. Gene therapies, using administration of nucleic acids rather than traditional drugs to patients, are being investigated for applications for a number of different diseases. Unlike traditional pharmaceuticals, gene therapy has the potential to treat almost any disease. Today, there are several approaches for correcting faulty genes: (1) inserting a normal gene into a nonspecific location within the genome to replace a nonfunctional gene; (2) replacing an abnormal gene with a normal gene through homologous recombination; (3) repairing an abnormal gene through selective reverse mutation; and (4) altering the regulation (the degree to which a gene is turned on or off) of a particular gene.

The concepts of gene therapy emerged in the early 1960s when genetically marked cell lines were first developed [1– 8] and when mechanisms of cell transformation by the papovaviruses polyoma and SV40 were identified [9– 11]. Since then, thanks to many scientific and technological breakthroughs in related fields, research on gene therapy has grown exponentially with significant advances observed in basic research as well as in patient care.

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Most of the early work on gene therapy focused on the delivery of DNA. However, in 1998, the role of double-stranded RNA in RNA interference (RNAi) was discovered [12], with Craig C. Mello and Andrew Fire being awarded the 2006 Nobel Prize in Physiology or Medicine. As a consequence of this path-breaking research, RNAi has become a major field of gene therapy, with both basic research and therapeutic applications being enthusiastically pursued.

The first gene therapy clinical trial in 1989 was for treatment of adenosine deaminase (ADA) deficiency using retrovirus [13,14]. Since then there have been more than 1034 US clinical trials [15]. To date, gene therapy has been used to treat a wide variety of diseases ranging from cancers [16– 18] to neurological diseases [19– 21]. The US is not the only country actively pursuing this therapeutic approach, and significant activities are occurring in countries all around the world [21]. In 2003, China became the first country to approve the commercial production and use of a gene therapy medicine: Gendicine, a recombinant Adenoviruses-p53 gene therapy for head and neck squamous cell carcinoma [22,23]. It is anticipated that continued research and clinical studies will lead to gene-based treatments for diseases such as ovarian cancer, cystic fibrosis, venous ulcers, hemophilia, glaucoma, as well as infectious diseases such as AIDS and graft-versus-host disease.

Unfortunately, there are many factors that have prevented gene therapy from becoming an even more wide-spread and effective treatment for disease. Some of the most notable reasons are listed below [24].

- *Limited membrane permeability of nucleic acids* – Since DNA, siRNA, and other nucleic acids are hydrophilic and negatively-charged macromolecules, they cannot efficiently cross cell plasma membranes, which contain the hydrophobic barrier of the lipid bilayer and typically have a net negative charge. While naked DNA can be introduced into cells through physical methods, such as electroporation [25,26], a “gene gun” [27], ultrasound [28,29], or direct injection into target tissue [30], the clinical relevance of these methods is limited, because naked DNA is susceptible to nuclease degradation in systemic circulation.
- *Short-lived nature of gene therapy* – Before gene therapy can become a permanent cure for a disease, the therapeutic DNA/RNA introduced into target cells must remain functional and the cells containing the therapeutic nucleic acid must be long-lived and stable. Problems with integrating therapeutic nucleic acid into the genome and the rapidly dividing nature of many cells prevent gene therapy from achieving long-term benefits.
- *Immune response* – Any time foreign material is introduced into human tissue, the immune system attacks the invader, often reducing gene therapy effectiveness. Furthermore, the immune system's enhanced response to previous treatments makes it difficult for the same gene therapy to be repeated in patients.
- *Multigene disorders* – Conditions or disorders that arise from mutations in a single gene are the best candidates for gene therapy. Unfortunately, some of the most commonly occurring disorders, such as heart disease, vasculature disease, Alzheimer's disease, arthritis, and diabetes are caused by the combined effects of variations in many genes. Multigene or multifactorial disorders such as these would be especially difficult to treat effectively using gene therapy today.
- *Problems with viral vectors* – Viruses, while the carrier of choice in most current gene therapy studies, present a variety of potential problems to the patients, including toxicity, immune and inflammatory responses, as well as issues with both

gene control and gene targeting. In addition, there is always the fear that the viral vector, once inside the patient, may recover its ability to infect its host.

Because viruses have been studied for many years as gene delivery systems, we now consider in more detail the potential strengths and limitations of this approach. All viruses bind to their hosts and introduce their genetic material into the host cell as part of their replication cycle. They have evolved for millions of years to become efficient carriers and, thus, have distinct advantages for delivery of gene into cells. Several different classes of viruses are used as gene therapy vectors. (1) Retroviruses create double-stranded DNA copies of their RNA genomes and copies of these genomes can be integrated into the chromosomes of host cells. (2) Adenoviruses are a class of viruses with double-stranded DNA genomes. The DNA molecule is left free in the nucleus of the host cell, and the instructions in this extra DNA molecule are transcribed just like any other gene. This vector system was employed in the first gene therapy product Gendicine. (3) Adeno-associated viruses are small, single-stranded DNA viruses that can insert their genetic material at a specific site on chromosome. (4) Herpes simplex viruses are double-stranded DNA viruses mostly examined for gene transfer in the nervous system.

Currently viral vectors are the most commonly used vectors in clinical trials and some have shown very promising results. Gene therapy trials using retroviral vectors to treat X-linked severe combined immunodeficiency (X-SCID), a fatal inherited disease, represent a successful clinical application of gene therapy to date. Nine patients who lacked an HLA-identical donor underwent *ex vivo* retrovirus-mediated transfer of γ chain to autologous CD34 + bone marrow cells between 1999 and 2002 [31,32]. After ten years, eight patients were alive after a median follow-up period of 9 years (range from 8 to 11). Transduced T cells were detected for up to 10.7 years after gene therapy. Seven patients had sustained immune reconstitution; three patients required immunoglobulin-replacement therapy. Sustained thymopoiesis was present, even after chemotherapy in three patients. Overall, correction of the immunodeficiency saved patients' lives and greatly improved their health [33].

Although viral vectors have shown success *in vivo*, safety has been the greatest concern. In 1999, an 18-year-old patient died from multiple organ failures four days after he started treatment in a gene-therapy trial for ornithine transcarboxylase deficiency (OTCD). His death was believed to have been triggered by a severe immune response to the adenovirus carrier. Another major incident occurred in 2002, when two children treated in an X-SCID gene therapy trial developed leukemia-like conditions.

Compared to viral vectors, non-viral or synthetic vectors have many advantages, such as low immunogenicity, low production cost, ease of modification, and better stability [34–46]. This makes nonviral vectors very attractive for gene therapy. However, most of the non-viral vectors suffer from low transfection efficiencies compared to viral vectors. That said, non-viral lipid vectors have been evaluated in the clinic, and Table 1 summarizes the currently open clinical trials using this system. In fact, the lipid – lipofectamine – has been used in about 7% of the worldwide gene therapy trials demonstrating the transition of this concept to the clinic. Therefore, a significant amount of research has focused on designing and developing new vectors that can overcome the transfection barriers and provide improved efficiencies. *One approach to solve this challenge is through the use of non-viral vectors that are functional or responsive to a biological signal and that will deliver their nucleic acid cargo in a timely and efficient manner. This review will focus on recent developments in responsive lipids and their corresponding lipid– DNA complexes (or lipoplexes) for improved DNA delivery*.

2. Barriers for gene delivery mediated by non-viral vectors

There are several steps in non-viral gene delivery, each of which can be a potential barrier (Fig. 1). Gene (DNA) delivery by non-viral vectors begins with DNA– vector complexation and formation of the lipoplex. The complex then must bind to the cell plasma membrane, enter the cell, traffic through the cytoplasmic organelles, and enter the nucleus where the DNA is released from the vector so that the DNA can reach the transcription machinery. The following section will briefly discuss each of these processes, the possible barriers, and some of the progress that has been made to date.

2.1. DNA complexation

The complexation of DNA with positively charged lipids or amphiphiles results from electrostatic interactions between the negatively charged phosphate backbone of DNA and positively charged molecules as well as hydrophobic forces between the aligned lipids. It has been shown that the size of the complex formed primarily depends on the type of cationic lipids used. Preparation conditions (such as concentration of DNA, pH, buffer composition, and salt concentration) usually lead to less dramatic changes, whereas the lipid cationic to nucleic acid phosphate anion ratio can have a significant affect. Typically, complexes are formed with a slight excess positive charge to permit them to interact with the negatively charged cell surface. The ratio between the cationic charge of the liposome and the negative charge of the DNA usually controls the size of lipoplexes [47]. At high positive charge ratios, relatively small complexes are formed (about 200 nm), whereas large aggregates (>1 μm) are usually formed when the net charge is close to neutrality [47,48]. However if a PEGylated lipid is used, relatively small lipoplexes of 100– 250 nm in diameter can be produced that have an overall neutral charge. Cationic polymers typically interact with DNA in a stronger manner, largely because of their multiple charges per molecule. Additionally, the molecular weight of the polymer can influence the size of the complexes. High molecular weight polylysine (224 kDa) forms DNA complexes with diameters ranging from 100 to 300 nm, while low molecular weight polylysine (~4 kDa) forms complexes with diameters between 20 and 30 nm [49].

Although advances have been made in structure– activity relationship studies, predicting *in vitro* and *in vivo* gene transfection efficiency based solely on the physicochemical properties of the complex is still not possible. Cellular uptake, endosomal escape, cytoplasmic movement, and nuclear targeting are all critical steps as described below.

2.2. Cellular binding

Unless a specific targeting ligand is present on the surface of the complexes, the binding of lipoplexes (and polyplexes) to the cell membrane is the result of a nonspecific ionic interaction between the positive charge of the complexes and the negative charge of the cell membrane. Negatively charged cell surface constituents (such as heparan sulfate proteoglycans and integrins) play a role in the cellular binding of positively charged lipoplexes or lipoplex formulation containing cationic peptides, such as TAT (GRKKRRQRRRPQ) [50– 53]. For example, in proteoglycan-deficient cells, the cellular binding of lipoplexes is reduced[54]. The heparan sulfate proteoglycans may act as non-specific receptors for cationic macromolecules, but their exact role in mediating cellular uptake is not clear. Some evidence suggests that the transmembrane proteins, syndecans, may cluster to form focal points at the plasma membrane during the binding process, with the cationic particle/clustered syndecan complex interacting with the actin cytoskeleton, resulting in the formation of tension fibers and initiation of cellular uptake. This process provides the energy required to engulf the particles [55].

2.3. Cellular uptake

Cellular uptake of non-viral vector/DNA complexes usually proceeds by endocytosis of the carrier[56– 58]. Five major endocytic pathways have been recognized [59– 63]. (1) Phagocytosis occurs in specialized cells such as neutrophiles or macrophages. (2) Macropinocytosis results when membrane ruffles fuse with the plasma membrane and form large endocytotic vesicles (up to 1– 5 μm) known as macropinosomes. They either fuse with lysosomes or are recycled back tothe cell surface. (3) Clathrin-mediated endocytosis occurs when ligand-bound receptors are internalized via clathrin-coated vesicles and subsequently transformed to early endosomes. (4) Caveolae-mediated endocytosis occurs by the evolution of caveolae-derivatives of the subdomains of sphingolipid and cholesterol-rich cell membrane fractions. (5) Clathrin- and caveolae- independent endocytosis includes those pathways not usually classified by the above criteria. The mechanisms of these pathways are actively investigated.

In terms of gene delivery by non-viral vectors, various endocytic uptake routes are usually involved, depending on whether or not targeting ligands are present. When considering non-targeted vectors, Rejman et al. reported that the uptake of DOTAP/DNA lipoplexes is inhibited by chlorpromazine and potassium depletion but is unaffected by filipin or genestein, suggesting that the uptake occurs solely by clathrin-mediated endocytosis [64]. Furthermore, they have shown that particles that are internalized by clathrinmediated endocytosis are eventually degraded in lysosomes. Similarly, Hoekstra et al. demonstrated that cationic SAINT/DOPE lipoplexes are taken up through the cholesterol-dependent clathrin-mediated endocytosis pathway in the COS-7, CHO, and HepG2 cells via a series of potassium depletion, cholesterol dependencies, and transferrin co-localization studies [65].

For non-targeting vector/DNA complexes, the size of the complex is an important parameter that affects cellular uptake in various cell lines. Amidon et al. showed that uptake of PLGA copolymer/DNA complexes in Caco-2 cells was size dependent with particles possessing a mean diameter of 100 nm showing the highest uptake [66]. Yao et al. reported that in four different cancer cell lines, PEI nanogels with mean diameters of 75 and 87 nm showed the highest transfection efficiency among complexes with six different diameters ranging from 38 nm to 167 nm [67]. Labhasetwar et al. also showed that cellular uptake of these same complexes in COS-7 and HEK-293 cell lines was higher for particles with mean diameters of 70 nm than those of 200 nm [68]. In another study that focused on either large particles (300– 700 nm) or small ones (50– 100 nm), it was observed that the larger particles were more effective than smaller analogues [69,70]. This result may be a consequence of “enforced” endocytosis by sedimentation of the large particles onto the cells in these *in vitro* studies.

With regards to targeted non-viral vectors, significant research has focused on developing vector systems with receptor ligands for delivery to specific cells or tissues. Some of these ligands are asialoglycoprotein, epidermal growth factor (EGF), folate, integrin, lactose, mannose, and transferrin [71]. Once recognized by the receptors on the plasma membrane, the targeted vector/DNA complexes are usually internalized through clathrin-mediated endocytosis. The effect of size on the cellular uptake of receptor-targeting vector/DNA complex may be more complicated than for non-targeting analogues. Aoyama et al. showed that internalization of glycocluster nanoparticles varies significantly with size when electrostatic interactions are excluded [72,73]. The optimal mean diameter for gene transfer was reported to be ~50 nm. This number was supported later by theoretical calculations performed by Gao et al. who determined the optimal size for particles to be 54– 60 nm [74]. Other studies show similar size-dependent variations in cellular uptake when asialoglycoprotein [75] or transferrin [76] are used as receptor ligands.

2.4. Endosomal escape

After internalization via endocytosis, the complexes exist in membrane-bound endosomes with no direct access to the cytosol or the nucleus. Moreover, endosomes generally either fuse with lysosomes for degradation or recycle their contents back to the cell surface. Therefore, escape from the endosome is essential for efficient transfection and several studies have addressed this problem using a variety of mechanisms.

In the case of a DNA– cationic lipid complex or lipoplex, disruption of the endosome could occur through the interaction with the cationic lipid by trans-bilayer flip-flop of anionic lipids from the external layer of the endosomal membrane [77]. This would lead to membrane destabilization and the release of naked plasmid DNA into the cytoplasm [78]. It has been demonstrated that cytoplasmic release of internalized lipoplex could involve charge neutralization of the cationic transfection agent with anionic macromolecules such as anionic membrane lipids and proteoglycans; cationic lipids mediated fusion; or membrane destabilization by pH-sensitive lipids, several of which will be discussed later [79– 81].

The composition and structure of the hydrophobic chains can affect lipoplex escape from the endosome, and this topic has been recently reviewed [46]. For example, decreased chain length or increased chain branching (or unsaturation) has been observed to afford higher transfection efficiencies, and this may be a consequence of greater intermembrane transfer rate and lipid mixing due to the lower phase transition temperatures of these lipids compared to their long-chain saturated analogs. This affect is nicely illustrated in a recent manuscript that compared the gene transfection efficiency of di-unsaturated versus mono-unsaturated lipid chain vectors [82]. Specifically, increased *in vivo* DNA transfection of mouse lung tissue using a plasmid DNA expressing the reporter gene luciferase via a tail vain injection was observed with a di-unsaturated compared to mono-unsaturated cationic lipophosphoramidate. Physicochemical studies revealed that the di-unsaturated cationic lipophosphoramidate had increased fluidity and fusogenicity characteristics, consistent with properties that would facilitate endosomal escape.

ATP-mediated proton accumulation makes the endosomal and lysosomal compartments of cells significantly more acidic (pH 5.0– 6.2) than the cytosol or intracellular space (pH ~7.4) [83]. Many non-viral vectors have been developed to utilize the acidic environment of endosomes and lysosomes to escape degradation. One method involves the incorporation of chloroquine into the DNA/vector complex. Chloroquine is a well-known lysosomotropic agent that raises the pH of the lysosomal environment thus inhibiting the enzymes involved in lysosomal degradation [84]. Alternatively, various macromolecules (such as polyethylenimine (PEI)) that have amine groups with low pKa values have been shown to have a “proton sponge” effect and are currently used in commercial in vitro transfection reagents. These compounds are capable of buffering the endosomal vesicle, which leads to endosomal swelling and lysis, thus releasing the DNA into the cytoplasm [85].

2.5. Cytoplasmic transportation

Once in the cytoplasm, DNA or DNA– vector complexes must overcome additional barriers in the cytosol to enter into the nucleus of the host cell. Dowty et al. were the first to discover that when plasmid DNA was microinjected into cytoplasm, passive diffusion was negligible and plasmid DNA remained predominantly at the site of microinjection [86], possibly due to cytoskeletal elements within the cytoplasm that function as molecular sieves and prevent the diffusion of macromolecules [87]. Viruses such as adenovirus serotype 5 [88] and herpes simplex virus [89] travel through the cytoplasm via microtubule-mediated transport. Cationic carrier mediated gene delivery lacks such means to be transported into nucleus.

DNA fragmentation in the cytoplasm represents another barrier. Cationic carriers may provide protection for DNA from such degradation in the cytoplasm. *In vitro* studies have demonstrated that complex formation dramatically increases the nuclease resistance of plasmid DNA [90,91].

2.6. Nuclear entry

The nuclear envelope represents a critical barrier for effective transfection. The nuclear envelope consists of a pair of apposing membranes containing nuclear pores with a passive transport limit of 70 kDa molecular mass or ~10 nm diameter [92]. This is much smaller than the size of DNA, even when condensed in lipoplexes. However, proteins (>20 kDa) can be transported into the nucleus in an ATP-dependent process triggered by reorganization of short peptide sequences that can be hindered by certain anti-nucleoporin antibodies and wheat germ agglutinin [93]. The expression of exogenous plasmid DNA can also be inhibited by wheat germ agglutinin, suggesting that gene transfer across the nuclear envelope proceeds via a similar pathway to proteins [86].

To enhance the nuclear uptake of DNA, nuclear localization peptide sequences (NLS) have been incorporated in gene delivery vectors [94]. In normal cell events NLS act to target proteins to nuclear pore complexes, where they are actively transported into the nucleus. It is important to note that the incorporation of NLS into gene delivery vectors does not necessarily protect the plasmid in the cytoplasm, and other elements may be needed to maximize nuclear delivery of intact plasmid vectors. An additional and complementary strategy being explored is to engineer the plasmid, which will be mixed with lipid, to contain recognition sites for transcription factors. For example, incorporation of a κB motif in a plasmid favors recognition and NFκB-driven nuclear import resulting in increased gene transfection activity compared to a plasmid lacking this motif [95].

2.7. Decomplexation of DNA– vector within the nucleus

A final barrier for gene delivery via non-viral vectors occurs within the nucleus. Direct injection of lipoplexes into the nucleus results in poor gene expression compared with injection of naked DNA [96]. This finding suggests that de-condensation in the nucleus can be a problem. Xu and Szoka proposed that the DNA is released from lipoplexes during endosomal release, thus delivering only naked DNA to the cytosol [97]. In contrast to cationic lipids, the microinjection of PEI polyplexes into the nucleus does not affect the transgene expression, suggesting that a rapid release of DNA from the polyplexes occurs in the nucleus, probably via an exchange with cellular DNA [96].

3. In vivo barriers for gene transfer

There have been many cationic DNA carrier systems that exhibit successful gene delivery in vitro, and it is evident that mechanisms controlling lipoplex binding and delivery to cells in culture are important to know and understand. However, successful *in vivo* use of non-viral vectors is even more complicated, as the complex usually has to be injected into the blood stream and enter into the circulation before reaching the cells of interest. Thus, some of the findings in the *in vitro* studies do not directly transfer to the *in vivo* studies. For example, optimal in vivo gene transfer (generally) requires a lower cationic lipid/DNA charge ratio than that used in cell culture [98– 100]. For *in vivo* applications, the physiological salt concentration of serum (150 mM) often promotes aggregation of the cationic complexes, which potentially could lead to vascular blockage [101]. Additionally, cationic complexes readily bind serum proteins (such as albumin), thereby hindering cellular uptake, promoting aggregation, and possibly inducing phagocytosis [102]. Serum protein association also plays a major role in the clearance of the lipoplex. For instance, liposomes consisting of egg

phosphati-dylcholine, cholesterol, and dioleoylphosphatidic acid (PC/CHOL/DOPA) which bind high levels of proteins are cleared more readily from circulation by macrophages than those containing dis-tearoylphosphatidylcholine and cholesterol (DSPC/CHOL), which bind much more poorly [103]. These results suggest that *in vivo* gene delivery can be improved by reducing salt/serum effects. The most successful method to date is to modify the complex with hydrophilic moieties, particularly poly(ethylene glycol) (PEG). The PEG forms a thin layer on the surface of the complex, which masks the cationic charges, reduces aggregation, and minimizes interactions with serum proteins. The range, magnitude, and interactive properties of these PEG steric barriers have been determined and modeled [104,105]. Particular emphasis was placed on analysis of lipids with covalently attached PEG2000 because that molecular weight PEG is found to optimize blood circulation times.

4. Functional lipid based vectors

The liposome was one of the earliest vehicles used to introduce exogenous genetic material into host cells. By 1980, several publications had demonstrated the capability of delivering exogenous DNA into cells using liposomes [106– 108]. Cationic lipids are typically composed of three structural domains: a cationic head group, a hydrophobic part, and a linker between the two domains. Some examples of commercially available lipid reagents include N-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethyl-ammonium chloride (DOTMA) [109], 2,3-dioleyloxy-N-[2(sperminecarboxamido) ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) [110], 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) [111], and dioctadecylamido-glycylspermine (DOGS) [50] (Fig. 2).

The mechanism of gene delivery by cationic lipoplexes has been reviewed [112,113] and it is now generally accepted that liposome-mediated gene transfer proceeds primarily through endocytosis [114– 116] . Following cellular uptake, lipoplexes can destabilize the endosomal membrane, resulting in a flip-flop reorganization of the membrane phospholipids. These phospholipids then diffuse into the lipoplex and interact with the cationic lipids causing the DNA to dissociate with lipids and to be released into the cytoplasm, as discussed above [77,97].

In the related area of delivery of drugs encapsulated in liposomes, numerous new methods and systems have been developed in order to improve drug encapsulation, retention, and stability of liposomes in blood circulation *in vivo*[117– 120]. A number of authors have provided evidence to support the hypothesis that a stimulus-responsive release mechanism can improve the efficiency and the specificity of liposomal drug delivery [121– 124]. For example, PEG-liposomes designed for optimal intravascular drug release in solid tumors by applied heat [125] are now in Phase III clinical trials. The greatest challenge is to optimize the location and time of drug release, which usually involves triggered release in response to the stimuli of the biological environment in the target cells or tissue. Similar challenges exist for the delivery of DNA. Thus, there are several key factors that must be considered in the design of a responsive or functional lipid for nucleic acid delivery. First, the stimulus to trigger the nucleic acid release must be specific or unique to the target site. Second, the complex must be sensitive enough to the trigger to yield effective DNA release. Third, the triggered release mechanism or component must be compatible with the other components of the complex to provide properties such as cargo retention, extended blood circulation time, deposition at the target site, protection of the DNA, cellular uptake, and delivery of the DNA to the nucleus.

The strategies developed to induce liposomal release of its cargo in response to an environmental stimulus include: 1) formation of defects and channels in the bilayer; 2) transition from a bilayer (lamellar) phase to a non-bilayer phase such as a micellar or

hexagonal phase; 3) lipid phase separation; 4) liposome fusion; 5) degradation of the lipid; and 6) reversing the electrostatic interaction between the lipid and nucleic acid [126–135]. The stimulus to induce release can be a biological one such as the drop of pH, enzymatic cleavage, or change of a redox potential. The chemical components that respond to these stimuli and induce the liposome leakage or release the cargo include ionizable lipids, lipids with a desired phase transition temperature (melting temperature, usually around physiological temperature), cleavable lipids, cis-trans isomerization, charge-switching lipids, and free-radical-generating compounds as photosensors. The following subsections will highlight several of the proposed mechanisms with specific lipid/lipoplex formulations. Within this group, the temperature sensitive drug delivery approach has been recently reviewed and, thus, this topic will not be discussed in detail [125,136–138]. It should be noted that a few of the studies cited below involve the release of small drug molecules encapsulated within the liposome. Additional complications may be involved in the release of large DNA molecules complexed to the component lipids.

4.1. pH-responsive lipids

A decrease in pH is implicated in many physiological and pathological processes such as endosome trafficking, tumor growth, inflammation, and myocardial ischemia [129,130]. Therefore, many pH-sensitive liposomes have been intensively studied over the past two decades for delivery of small molecules, macro-molecules, or biomacromolecules [128,129,131,132].

The mechanisms of pH-triggered liposome destabilization include: 1) neutralization of negative lipids in the bilayers via protonation, leading to a lamellar to hexagonal phase transition; 2) protonation of negative polymers or peptides, which in turn adsorb to the bilayer and destabilize the bilayer structure by lysis, phase separation, pore formation, or fusion; 3) acid-catalyzed hydrolysis of bilayer-stabilizing lipids into destabilizing detergents or conical lipids; and, 4) ionization of neutral surfactants into their positive and surface-active conjugate acids [139].

Before they can achieve sufficient accumulation at the target site, the liposomes need to be reasonably stable in the circulation, avoiding RES uptake or rapid clearance. However, the sensitivity of the liposomes to the change in pH needs to be sufficient to respond to the decrease of pH at target sites. For example, the trafficking through the endosome in cells occurs within about 10–30 min with pH decreases from ~7.4 to 5–6. After that, the endosomal contents fuse into the lysosome where extensive degradation takes place [140]. Therefore, the pH-sensitive liposomes must respond to the initial drop in pH and release their contents relatively quickly to avoid lysosomal compartmentalization. At inflammatory tissues [141] and solid tumors [142], the pH is only 0.4–0.8 units more acidic than that of the blood stream, which means that liposomes designed for these purposes need to respond to a small stimulus and release enough nucleic acid for a therapeutic effect. It should also be noted that the requirements may vary depending on the therapeutic agent delivered. As noted above, in the case of small drug molecules, triggered release within the blood stream or interstitial space of the targeted tissue may be sufficient, provided that the surrounding cells can readily take up the drug. However, for delivery of biomacromolecules, such as nucleic acids, release must happen after the lipoplexes cross the plasma membrane.

4.1.1. Neutralization of liposome bilayers—The first pH-sensitive liposome system was composed of phosphatidylcholine and *N*-palmitoyl homocysteine (PHC). Upon exposure to lower pH, PHC undergoes a transition from a charged open form to an uncharged thiolactone ring form, which destabilizes the bilayer and releases the encapsulated drug cargo [143]. Building upon this initial work with PHC, a variety of

liposomes that possess a pH-titratable carboxylate group and a fusogenic lipid such as DOPE have since been studied [131]. The decrease of pH results in the neutralization of the excess negative charges of the carboxylate groups, which reduces the surface area of the head group and triggers the transition of the PE-rich lamellae into a hexagonal phase with concomitant release of the encapsulated contents. However, at neutral pH, the excess negative charges of the carboxylate groups on the liposome surface may induce undesired interactions with plasma proteins and fixed macrophages, leading to rapid elimination of the liposomes from circulation [121].

4.1.2. Hydrolysis of liposomes—To circumvent potential problems due to the negatively charged surface of pH-sensitive liposome, cleavable liposomes with noncharged functional groups whose hydrolysis is catalyzed by acidic conditions have been designed. Different head groups, lipid chains, linker groups, and linkage configurations can be introduced to generate various structures of desired properties, and Cordes and Bull have described several strategies [144]. Several pH-sensitive functional groups are discussed below. Finally, the hydrolysis of liposomes by phospholipase A2 (PLA_2) is described.

Thompson et al. reported a number of lipids with an acid-sensitive vinyl ether linkage between the head group and the hydrocarbon chains (Fig. 3) [145–147]. Upon exposure to low pH, the vinyl ether chains are cleaved leading to structural defects in the bilayer and the release of the encapsulated contents. At 38 °C, liposomes composed of plasmenylcholine (1-hexadecyl-1*Z*′-enyl-2-palmitoyl-*sn*-glycero-3-phosphocholine) need about 4 min to release 50% of their contents at pH 2.3 and about 500 min for 50% release at pH 5.3 [148]. However, while the incorporation of dihydrocholesterol into the bilayer improves its serum stability, it also greatly reduces the pH sensitivity: at pH 2.3, over 70 min are needed to effect 50% content release in 6:4 PlsPamCho/DHC liposome. Liposomes composed of diplasmenyl phosphocholine (DPPlsC) possessed better pH sensitivity and released 50% of encapsulated calcein in 230 min at pH 5.3 [149]. When KB cells are treated with folate targeted DPPlsC liposomes (DPPlsC:DSPE– PEG_{3350}– folate = 99.5:0.5) containing propidium iodide (PI), 83% of the PI escape the endosomal/lysosomal compartments within 8 h. Encapsulation of 1-β-arabinofuranosylcytosine into these liposomes enhances its cytotoxicity in KB cell culture by 6000-fold compared with the free drug. These results in cell culture nicely demonstrate the principle that the introduction of a pH-triggering mechanism into targeted liposomes can control delivery and significantly increase the efficacy of the encapsulated therapeutic agents.

Further development produced O-(2R-1,2-di-O-(1′Z, 9′Z-octa-decadienyl)-glycerol)-3-*N*-(bis-2-aminoethyl)-carbamate (BCAT) which undergoes complete hydrolysis in acidic solution. Use of this lipid afforded higher levels of transgene expression in comparison to the non-hydrolysable saturated diether analog DCAT. After IV administration, mice treated with BCAT did not exhibit any obvious signs of toxicity, whereas all the mice treated with DCAT died [147]. The toxicity of the non-hydrolysable DCAT is consistent with earlier observation that the more stable ether linked lipids are more toxic than ester linked vectors, which may be more easily cleaved within the cell [150,151].

Liposomes containing the acid-labile acetal linkages have also been studied [152– 155]. Song and Hollingsworth [154] reported a glycol lipid conjugate of glucose with two palmitoyl chains connected via an acid-labile acetal moiety (Fig. 4). The glycol lipid selfassembles into lamellar structures in aqueous solution and the acetal linkage is completely cleaved in ethanol solution with 0.01% concentrated DCl (pD slightly lower than 3), but no cleavage is observed when the lipid is in ethanol with 1– 20% acetic acid. It remains to be seen if the kinetics of the hydrolysis of this glycol lipid is sufficient for applications in triggered drug release *in vivo*. Zhu et al. studied the steroid-based lipid

cholest-5-en-3-one 3-(dimethylammonium chloride)propylene acetal (Fig. 4) with an acid-sensitive acetal bond, which undergoes hydrolysis in acidic medium where an ether analogue remains undegraded. It achieves levels of gene delivery similar to DC-Chol [155].

Compared to vinyl ethers and acetals, ortho esters are more acid-sensitive functional groups [156]. They can hydrolyze more quickly in response to pH decrease, due to the stable dialkoxy cation intermediate as shown in Fig. 5 [144]. Two cationic lipids (Fig. 6) containing an ortho ester linker based on the structure of 3,5,8-trioxabicyclo[2.2.2]octane were synthesized and characterized by Zhu and co-workers [155]. However, the first two fast hydrolysis steps only add two hydroxyl groups near the cationic head group region. Thus, the lipid converts to two single-chain compounds only after the cleavage of an ester group, which is the final and slower step of the hydrolysis. Such a hydrolysis pattern complicates the kinetics of bilayer destabilization by these lipids and may affect delivery of DNA. Recently, Chen et al. synthesized two cationic lipids, which contain a cationic head group and an unsaturated hydrophobic dioleoylglycerol moiety joined together by a linear or a cyclic ortho ester linker (Fig. 7) [157]. At pH 7.4, the lipids form stable lipoplexes with plasmid DNA in the presence with helper lipid DOPE. With decreased pH, the hydrolysis of the ortho ester linkers removes the cationic head groups and causes the aggregation of the lipoplexes. At pH 5.5, the cationic lipid with the cyclic ortho ester linker shows exceptional pH sensitivity and the aggregation takes place within 32 min. The endosomal pH 5.5 triggers its lipoplex to induce a rapid leakage (70% in 30 min) and permeate model biomembranes within the time span of endosome processing prior to lysosomal degradation. The lipid shows significantly improved transfection efficiency in CV-1 and HTB-129 cells compared to the pH-insensitive control lipid DOTAP.

Despite the vast diversity of pH-sensitive liposomes, success of these systems for *in vivo* drug or gene delivery can be limited due to interactions with serum. Guo and Szoka designed and synthesized a lipid conjugate (Fig. 8) of PEG2000 and distearoyl glycerol via an acid-labile diortho ester linker [158]. PEG2000 was chosen as the head group because it is stable *in vivo* and, as noted above, this size of PEG provides a prolonged blood circulation time [117]. The 3,9-diethyl-2,4,8,10-tetraoxaspiro[5,5] undecane moiety was chosen as the diortho ester linker, based on its pH sensitivity and biocompatibility in related polymeric drug delivery systems [159]. The first step of the hydrolysis of either of the two ortho ester groups leads to the immediate cleavage of the PEG head group from the conjugate. When it is formulated with unsaturated phosphatidylethanolamine, distearoyl glycerol will be generated upon hydrolysis and will favor the formation of hexagonal phases due to its conical structure. The PEG2000-ortho ester-distearoyl glycerol conjugate (POD) is relatively stable in pH 7.4 buffer at 37 °C but degrades completely within 1 h when the pH is decreased to 5. POD/DOPE liposomes (1/9 in molar ratio) are as stable as the sterically-stabilized and pH-insensitive control liposomes (DSPE-PEG2000:DOPE = 1:9) in serum for up to 12 h. However, when POD/DOPE liposomes are incubated in acidic pH as mild as 5.5, they aggregate and release most of their contents within 30 min, a timeframe compatible with endosome trafficking at this pH [140]. Upon IV injection into mice, the POD/DOPE liposomes are eliminated from circulation with a half-life of 200 min, which is comparable to the DSPE-PEG2000/DOPE liposomes. The fast degradation kinetics of POD at low pH and liposome stability in blood circulation may provide considerable advantages for triggered drug and gene delivery in mildly acidic bio-environments such as endosomes, solid tumors, and inflammatory tissues.

Lipid structures that are susceptible to mildly alkaline conditions have also been devised, not for pH-triggered release but rather for pH-triggered assembly of DNA delivery systems. Ouyang et al. synthesized a series of novel cationic surfactants that contained a quaternary amine group and a cleavable hydrophilic isothiuronium head group (Fig. 9) [160]. They

were used to control the assembly of plasmid DNA into small stable particles with high DNA concentrations. The hydrophilic isothiuronium head groups provide relatively high critical micelle concentration (CMC) (>10 mM). The isothiuronium group masks the sulfhydryl group on the detergent and can be cleaved in a controlled manner under basic conditions. After the surfactants accumulate on a DNA template, the unmasked sulfhydryl groups dimerize to form disulfide-linked cationic lipids containing two alkyl chains. A ~6 KB plasmid DNA can be compacted into a small particle with an average diameter of around 40 nm and a – 13 mV zeta potential at high DNA concentrations (up to 0.3 mg/mL). Under appropriate conditions, the small particles retain transfection activity.

Hydrolysis of the lipids can also occur via an enzymatic reaction leading to degradation of the liposome and release of the encapsulant. For example, dipalmitoylphosphatidyl choline (DPPC) lipsomes can be degraded in the presence of phospholipase A2 (PLA2). Thompson et al. reported an elegant example of using PLA_2 to catalyze the release of an encapsulant [161]. In their study, a solution containing two different types of liposomes was used where photolysis of diplasmenylcholine liposomes containing bacteriochlorophyll in the membrane afforded release of the entrapped calcium which then activated the calcium-dependent PLA_2 in solution to catalyze DPPC hydrolysis in a secondary reaction yielding release of the encapsulated calcein. More recently, Foged et al. reported a liposomal carrier for release of siRNA in the presence of secretory phosholipase A2 (sPLA2) [162]. sPLA2 is present in upregulated levels at sites of inflammation and cancer and, thus it can be used as a trigger for site-specific delivery from a degradable liposome. Specifically, liposomes composed of DPPC, dipalmitoyl-phosphatidylglycerol (DPPG), and DPPE-PEG20 were prepared containing siRNA directed against EGFP for evaluation in HeLa cells. The anionic DPPG lipid was used since this would impart a negative charge to the liposomal surface, and it is known that sPLA2 activity is optimal when it is at an anionic surface interface. The siRNA-loaded liposomes are readily taken up by HeLa cells and the siRNA is localized in vesicular compartments, however EGFP expression is not silence. The lack of gene knockdown is attributed to confinement of the siRNA to the endosome, as the siRNA must be in the cytoplasm to be active.

4.1.3. Ionization of liposome structures—In an effort to improve the gene transfection efficiency of cationic liposomes, Liang and Hughes [163,164] designed biode-gradable, pH-sensitive surfactants as potential lysosomotropic agents. These surfactants possess a single 12-carbon alkyl chain and a pH-titratable imidazole group that becomes cationic at acidic pH. Liposomes composed of PC and these pH-sensitive surfactants undergo fusion and release their cargo in a manner which depends on both pH and the molar ratio of BPS to membrane lipids. Among the three surfactants systems reported, dodecyl 2-(1′-imidazolyl) propionate (DIP, Fig. 10) shows the highest pH-sensitive leakage, releasing more than 40% of the encapsulated calcein at pH 5 and about 10% at neutral pH. The incorporation of cholesterol into the lipid composition significantly enhances the pH sensitivity, whereas the presence of DOPE decreases the pH sensitivity. pLG3 plasmid DNA delivered by cationic liposomes composed of DOTAP, DOPE and DIP (1:1:1 molar ratio) shows 5-fold more luciferase gene expression in a human neuroblastoma cell line SKnSH, compared with the control formulation of DOTAP/DOPE in 1:1 molar ratio. However, the use of the pH-sensitive surfactants for *in vivo* applications is hampered by the propensity of the single-chain surfactant to readily transfer from liposomes into biological membranes such as red blood cell membranes, as illustrated in a study by Asokan and Cho [130]. They reported a series of acyloxyalkyl imidazole lipids that can induce hemolysis in a pH-dependent manner. Among the reported lipids, myristoyloxymethylimidazole (Fig. 10) at pH 5.5 completely disrupts human erythrocytes within 10 min, whereas at pH 7.0 and above, 50 min is needed for complete hemolysis.

Lipids containing imidazole or histidine functionalities have also received significant attention, and this area has been recently reviewed by Midoux and Jaffrès [165,166]. Of these, the histidine lipids belong to the family of bio-inspired peptide-based lipids which are actively being investigated [167– 169]. One of the first examples of a pH-sensitive lipid was by Budker et al. who synthesized lipid A as shown in Fig. 11 [170]. Once the imidazole is protonated at mild acidic pH conditions, lipoplexes can be formed in the presence of DOPE. Compared to a non-functional lipoplex prepared with DOTMA/DOPE, the lipid A/DOPE formulation gave higher transfection efficiencies in several cell lines including NIH 3T3 and HepG2 cells [170]. Chaudhuri et al. have reported a library of such cationic lipids for gene delivery including lipid B (Fig. 11) [171– 174] . Besides these histidine amphiphiles being active carriers for DNA in CHO, 203T7, A549, and HeLa cells, the lipids possessing multiple histidines transfected DNA in the presence of serum. Using a phosphoramidite as the building block, Mevel et al. synthesized histidine lipids such as the one shown in lipid structure C (Fig. 11) that are neutral at pH 7.4 [175]. When used as a helper lipid and combined with a cationic lipid, the resulting lipoplexes show 100-fold enhancement in gene transfection compared to a similar formulation with DOPE. As such, the helper lipid confers new properties to the lipoplexes to include stabilization of the aggregates at pH 7.4 and increased fusogenicity at pH 6.0 when in the endosome. At pH 7.4 the imidazole possesses one hydrogen bond acceptor and one donor site and thus can form a hydrogen bonded network, whereas at pH 6.0 this hydrogen bond network is likely perturbed due to the protonation of the imidazole. It is this protonation of the imidazole moiety that affords increased fuso-genicity of the lipid at pH 6.0.

4.2. Charge-reversal or charge-switching lipids

Grinstaff et al. reported a functional lipid for gene delivery that undergoes an electrostatic transition intracellularly from cationic to anionic to improve the release of DNA from the lipoplex [133,176]. The charge-reversal lipid **1** has a cationic ammonium head group to bind DNA, lipophilic acyl chains to form a bilayer, and benzyl esters at the terminus of the acyl chains for enzymatic hydrolysis (Fig. 12). This charge-reversal lipid binds DNA when cationic and then releases DNA when it is anionic. As an anionic multi-charged amphiphile it can also destabilizes bilayers. Thus, the lipid undergoes the following reactions: it complexes plasmid DNA and forms the lipoplex of ≈ 250 nm in diameter; upon entering the cell esterases hydrolyze the terminal ester linkages to produce anionic amphiphiles; and finally, the anionic amphiphiles repel DNA and disrupt the lipid bilayer of the supramolecular complex releasing the plasmid DNA for subsequent transcription. To assess the role of each structural component, compounds **2**– **4** were also prepared and characterized (Fig. 13). As expected, all the compounds bind DNA except for the anionic amphiphile **2**, the product of enzymatic hydrolysis of compound **1**. Transfection experiments using the reporter gene β-galactosidase were performed with CHO cells and showed that the cationic lipid **1** exhibits the highest transfection efficiency with activity better than the positive controls DOTAP and TransFast™. Significantly, compounds **2** through **4** show minimal transfection activity, consistent with their poor affinity to bind DNA. The lack of transfection with **3** conveys the important role the cleavable terminal ester linkages perform in this lipid. In addition, mechanistic studies using fluorescence co-localization techniques and endocytosis-interfering drugs indicate that macropinocytosis is the major pathway leading to DNA transfection in CHO cells with compound **1** [177]. This research has also led to a series of new charge-reversal lipids which possess different head-group spacer compositions revealing that more rigid spacers perform better [178].

Building upon these results, Grinstaff et al. synthesized and studied a charge-reversible helper phospholipid composed of a zwitterionic phosphatidylcholine head group, two long acyl chains, and ester linked benzyl terminal group [179]. This lipid forms vesicles of 127

nm and 106 nm average diameter in the presence and absence of DOTAP, respectively. X-ray diffraction patterns obtained of the lipid/DOTAP/DNA complex (1:1 ratio lipid to DOTAP) show a 15 Å increase in the lamellar repeat period compared to lipid only vesicles, indicating the formation of multilamellar structures with DNA sandwiched between bilayers. This type of structure has been observed previously for DNA– DOTAP and phosphatidylcholine complexes [180,181]. Increased transfection activity in CHO cells by 4 fold is observed compared to the DOPE/DOTAP control system.

Whereas in the above examples intracellular esterases enzymatically activate the charge-reversal effect, Lynn et al. approached the problem through chemical methods by inducing reversible reduction and oxidation of ferrocene-based cationic lipids to alter the charge of the lipid. Specifically, a bis-(11-ferrocenylundecyl) dimethylammonium bromide (BFDMA) lipid that contained two chains with ferrocene terminal groups attached to a cationic head group was prepared following the work of Abe who studied the vesicles/micelles formed by this lipid based on the charge of the lipid [182,183]. The ferrocene moiety allows for reversible reduction and oxidation by chemical methods (i.e., glutathione as a reducing agent). The reduced form of BFDMA (+1) forms bilayer vesicles whereas micellar aggregates are formed with the oxidized (+3) compound. The oxidized (+3; ferrocenium containing BFDMA) cationic lipids are poor transfection vectors for plasmid DNA in simian kidney (COS-7) cells [184,185]. However, the reduced (+1) form of these BFDMA lipids is more effective in gene transfection with activity increasing with longer lipoplex incubation times (2 h, 4 h, 12 h). Importantly, the reduced BFDMA lipid transfects DNA in serum-rich media.

Next, they studied the effects of a reducing agent, glutathione, on the transfection activity of these redox-active ferrocene-based lipids [186]. Addition of glutathione changes the ferrocenium group in the oxidized BFDMA to the reduced form (+1) in both solution and within lipoplexes. The rate of this reduction varies from 90 min with a 10-fold excess glutathione added, to 100 s with addition of a 50-fold excess. Zeta potential measurements show that the negative surface charge of the lipoplexes when formed with oxidized BFDMA switches to a net positive surface charge after incubation with glutathione. To evaluate the effects of added glutathione on transfection in the presence of this lipid (COS-7 cells), they performed experiments with luciferase encoding plasmid with the different BFDMA lipids in the presence and absence of the reducing agent. As expected, the reduced BFDMA shows substantial transfection, while the oxidized BFDMA alone has minimal activity. The glutathione-treated BFDMA lipoplexes show transfection efficiencies comparable to or greater than those of synthetically prepared reduced BFDMA. Cellular internalization studies reveal that both the reduced BFDMA and glutathione-treated BFDMA lipoplexes co-localize in the endosome and in the cytosol, but the oxidized BFDMA ones do not.

4.3. Reducible lipids

The next approach entails the incorporation of reducible linkages within cationic lipids to facilitate lipoplex destabilization, breakdown, and DNA release. The rationale behind this approach is that the cytoplasm has relatively high (10^2– 10^3 fold difference) [187] concentrations of reductive species, such as 10 mM glutathione [188] and the enzymes thioredoxin and glutaredoxin [187]. Once internalized in the cell, the lipoplex possessing a redoxsensitive disulfide bond within its structure will undergo S– S cleavage. In this scenario, reduction of the disulfide bond affords degradation of the lipid, release of DNA, and destabilization of the liposomal membrane. Two general structural designs are utilized: the reducible linkage connects the head group to the acyl chains or it is located within the hydrophobic chains.

Hughes et al. have synthesized and investigated cationic lipids containing disulfide bonds for gene delivery. Lipoplexes prepared with 1,2-dioleoyl-sn-glycerol-3-succinyl-2-hydroxyethyl disulfide ornithine lipid (DOGSDSO), DOPE, and DNA (luciferase gene) afford a 50-fold increase in transfection in human embryonic kidney (HEK 293) cells and simian kidney (COS-1) cells and a four-fold increase in human neuroblastoma (SKnSH) cells when compared to the nonreducing analog (DOGSHDO; Fig. 14) [189]. The functional vector undergoes reduction and release of DNA in the presence of DTT, but with glutathione, a naturally occurring intracellular reducing agent, DNA release was less. Consequently, a more readily reducible cationic lipid containing a dithiodiglycolyl linker (CHDTAEA) was prepared, as shown in Fig. 14 [190]. The two electron withdrawing carboxyl groups near the disulfide in DOGSDSO increase the rate of reduction. This lipid forms lipoplexes that release their DNA upon the addition of glutathione, displays 7 fold greater transfection efficiency in SKnSH cells, and is relatively non-toxic [190,191].

Based on the natural amphiphile lipoic acid, Balakirev et al. have designed and prepared a compound capable of both intra- and intermolecular disulfide bond formation via the thermodynamically unstable 1,2-dithiolane ring, which undergoes thiol-disulfide exchange and self– polymerization reactions [192]. The monomeric or polymeric forms of the bis-dithiolane AP1 (*N*-[1-(2,3-dilipoyloxy) propyl]-*N,N,N*-trimethyl ammonium methylsulfate) (Fig. 14) are reduced by DTT to form tetrathiol compounds that exhibit a lower binding affinity for DNA, thereby endowing the system with a triggered DNA release mechanism. Using electron microscopy, it was observed that in the oxidized state, the lipid condenses DNA into spherical lipoplexes; upon reduction, the lipoplexes swelled and eventually disappeared. Importantly, the DNA release by endogenous reducing agents requires the higher concentrations found in the nucleus rather than the lower ones found in the cytoplasm. *In vitro* transfection show only slightly better gene transfection with lipid AP1 in its polymeric form compared to DOTAP. However, when the vector was polymerized in the presence of the adenovirus fiber peptide pI, up to 6– 10 fold higher transfection levels are observed.

The effect of the disulfide group position in the cationic lipid has also been studied. Scherman et al. prepared a series of lipopolyamines that contain reducible disulfide bonds at various structural positions within the lipid in order to evaluate its consequence on transfection [193,194]. In one example, the disulfide group is located in the linker domain and in the other between the linker and an additional C5 or C12 hydrocarbon chain (Fig.15) [193,194]. *In vitro* transfection experiments showed that the vector with the disulfide located in the linker domain results in a loss of activity, whereas the lipids with a disulfide connecting the additional side chain displays a substantial improvement in transfection efficiency. Also, cleavage of one of the two fatty acid chains, which converts the lipid into a detergent, also led to higher transfection compared to a non-cleavable analogue. In a subsequent study [194] they synthesized additional vectors (shown in Fig. 16) including: 1) a symmetric lipid with the disulfide bond between the hydrophilic region and hydrophobic region (DSL-1); 2) a gemini lipid with the disulfide connecting the two identical lipopolyamines (DSL-2); 3) an asymmetric lipid with one short alkyl chain and 1 long alkyl chain that contains the disulfide bond (DSL-3); 4) a similar lipid to DSL-3 but with a long alkyl chain and the disulfide located in the short alkyl chain (DSL-4); and the non-reducible counterpart to DSL-3 (NDSL). All of the reducible lipids complex DNA and form lipoplexes with similar size and charge ratios to conventional lipoplexes, as determined by dynamic light scattering [194,195]. Small lipoplexes (<200 nm diameter) are formed at charge ratios <1, whereas large aggregates (~600 nm– 800 nm) are formed at charge ratios near 2. At charge ratios greater than 4, small lipoplexes (<200 nm) are again observed. Addition of the chemical reducing agent dithiothreitol cleaves the disulfide bonds within all of the lipoplexes, with the reaction being most efficient when the disulfide linkage is in a

hydrophilic region of the lipid. In addition, those lipids within lipoplexes that lost significant portions of their hydrophobic domains after reduction (DSL-1 and DSL-3) release DNA, whereas the other lipoplexes either release some or none of their DNA. Transfection experiments delivering the luciferase gene (pCOr plasmid pCMV-Luc) to HeLa cells show that the reducible lipoplexes (DSL-1, DSL-3, and DSL-4) exhibit a 1000-fold increase in transfection efficiency compared to their non-reducible counter-parts (NDSL) [194].

Despite the improved activity and lower toxicity of these redox-responsive cationic lipids, the cellular mechanism of disulfide reduction is not yet fully understood. One possible mechanism is that small molecules with free sulfhydryl groups, such as glutathione and cysteine, which exist predominantly inside the cells [196] can trigger the release of DNA from the liposomes. However, since most liposomes are taken up by cells via endocytosis, it is still unknown whether the liposomes encounter these reducing agents in the endosomal compartments or at other times during the transfection pathway. Moreover, the endosomal compartments normally go through an acidification process, and disulfide reduction is less favored at lower pH values. Recently, the discovery of the first reducing enzyme GILT (gamma-interferon-inducible lysosomal thiol reductase) located in the endosomal pathway was reported, providing a possible mechanistic route to reductive lipid degradation [197]. Another possible mechanism involves membrane-bound reductive enzymes. Ryser et al. have demonstrated that protein disulfide isomerase (PDI), a chaperon enzyme present in the ER and the plasma membrane of eukaryotic cells, plays a pivotal role in the thiolysis of macromolecules that do not diffuse through biomembranes [198]. Inhibition of PDI eliminates the activity of disulfide-containing toxins such as diphtheria toxin [199]. Thus, disulfide-containing liposomes may be triggered by PDI-catalyzed thiolysis after attaching to the cellular surface.

5. Conclusions

It has been over forty years since the concept of gene therapy was first introduced. The study of gene therapy has advanced from basic scientific research to clinical applications. Although it has gone through periods of more and less intense activity, gene therapy is showing significant promise as an emerging therapy for many ailments, some of which are currently impossible to treat or cure by traditional medicines. Despite the promising success of gene therapy, major hurdles still remain for the delivery of genes into target cells or tissues. Both viral and non-viral vectors are investigated as gene carriers and many clinical trials have been and are being performed. Due to their infectious nature, viral vectors show high transfection efficiency both *in vitro* and *in vivo*. Although viral vectors are now in commercial production and used in the clinic, non-viral vectors do offer many advantages compared to viral vectors. However, they generally suffer from low transfection efficiency. Thus, a significant fraction of current research on non-viral vectors focuses on developing new approaches to improving transfection efficiency *in vitro* and *in vivo*. Among the non-viral vectors investigated so far, functional or stimulus-responsive vectors are of special interest, since these vectors undergo physical or chemical reactions in response to changes in pH, oxidative state, or enzymatic activity. As these stimuli or cues may be specific to a biological site, tissue, or condition, it may facilitate the release of the nucleic acid cargo at the desired site in an efficient manner. Although many such vectors have shown success *in vitro* and a few *in vivo*, none have entered clinical phase. In our opinion, the primary reason is insufficient transfection activity.

To overcome this barrier of low transfection efficiency, significant activities and resulting accomplishments on the synthetic, mechanistic, and biological fronts are needed. Once we are successful, there will be a number of other real and practical challenges that will need to be completed such as large scale lipid manufacturing and lipoplex formation, shelf-life, and

stability, but we will sideline this discussion until a later date. That is not to say that we should not be mindful of these barriers. On the synthetic front, we have limited ability to predict lipoplex structure and transfection efficiency based on discreet changes in the chemical structure of the lipid. Additional systematic studies are needed. Although it is informative to investigate the modifications of a single structural part of any cationic lipid, the properties of the lipid/DNA complex as a whole affect transfection efficiency. These characteristics include but are not limited to the type and number of interactions with DNA, the hydrophobic/hydrophilic balance, complex size, surface charge, physiological stability, and mechanism of DNA release from the carrier. And we must be able to characterize these properties using an array of analytical techniques. All of these appear to affect cellular binding, uptake, intracellular trafficking, protein expression, biodistribution, serum stability, and so forth. Thus, a systems approach for analysis and discovery is needed where one examines a specific chemical entity(ies) or biological reaction(s) as well as the cooperative interaction(s) of the different constitutive parts. As it may be difficult to incorporate diverse functions required to overcome all the delivery barriers in a single cationic lipid, the development of sophisticated, multimodular DNA carriers – which incorporate multiple functions to maximize the transfection efficiency – are actively being pursued. Although this is a viable strategy, we are less enamored with this approach as increased vehicle complexity leads to increased difficulty. Meaning, if a synthetic vector has seven different lipids/components each of which performs a unique role, the synthesis and analytical methods, the formulation and its reproducibility, and the mechanism of action all need to be defined and understood. Obviously, from the regulatory and clinically point of view, a simpler system with equivalent performance would likely be favored. Therefore, a synthetic vector for clinical applications should balance these sometimes conflicting requirements with satisfactory *in vivo* performance, safety profile, formulation, and ease of use.

Through rational design, structure– activity relationship studies, and mechanistic investigations, improvements in non-viral vectors will be forthcoming, and these successes will enable efficient delivery with therapeutic outcomes and improved patient care. It is a challenge that all of us in the field must strive for. Advancement in functional vector design is one approach that combined with targeted or localized delivery may lead to the next generation of synthetic vectors. In summary, functional or stimuli responsive vectors offer a myriad of opportunities for basic studies of nucleic acid– lipid interactions and structure as well as for in vitro and *in vivo* use to address specific clinical applications.

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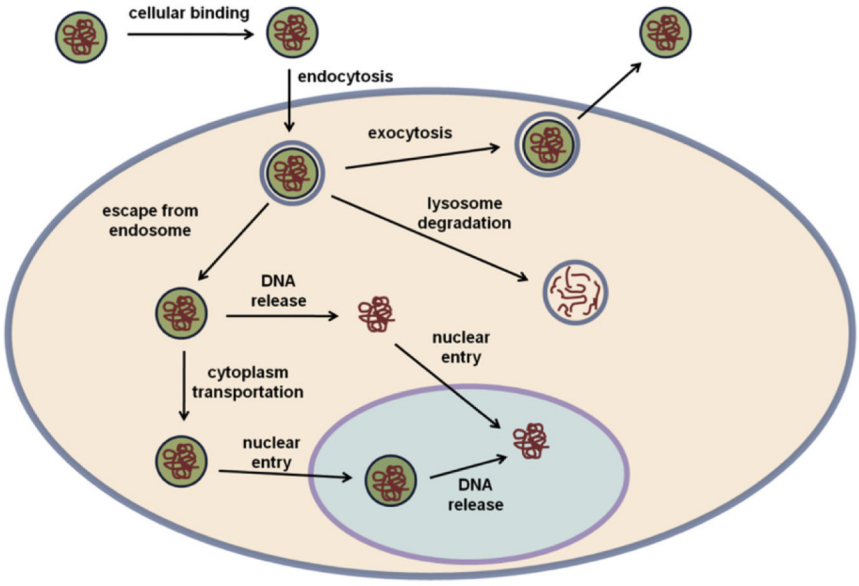


Fig. 1.
Intracellular barriers of gene delivery.

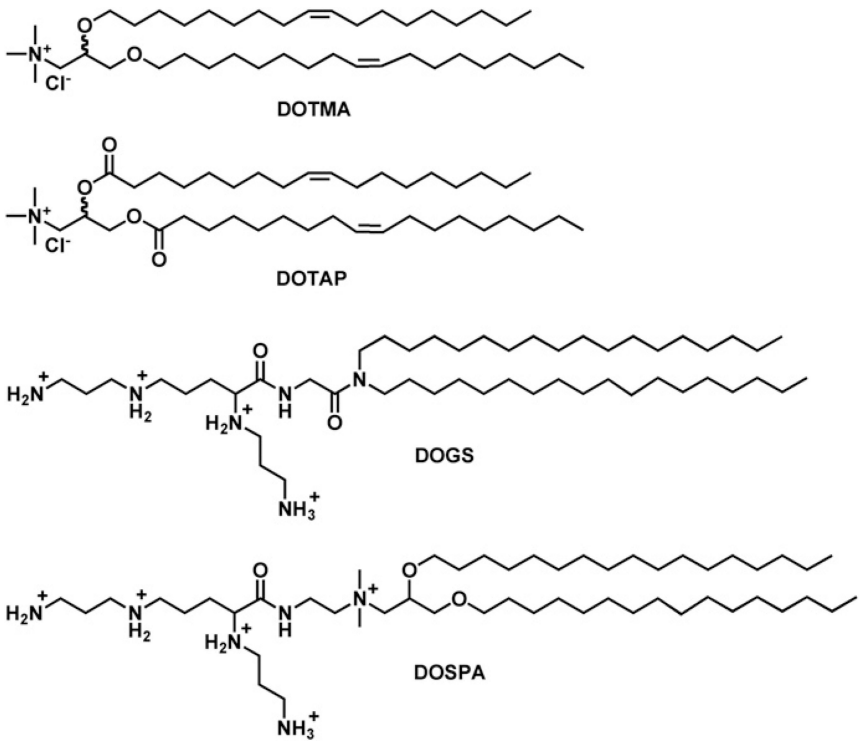


Fig. 2.
Examples of commercially available lipid reagents.

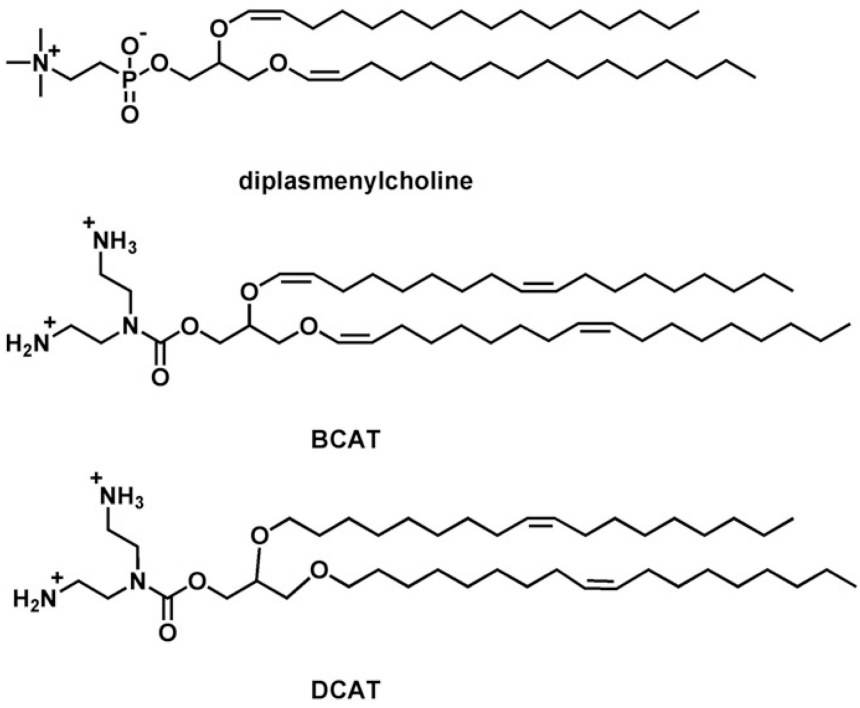
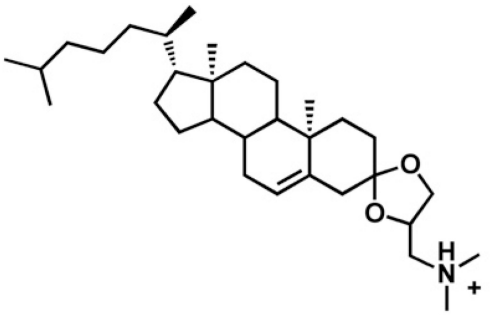
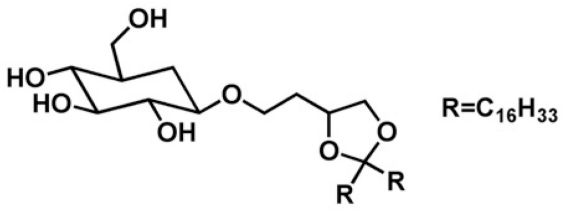


Fig. 3.
Acid-sensitive lipids with vinyl ether linkage (diplasmenylcholine and BCAT) and one non acidic-sensitive diether DCAT lipid.



cholest-5-en-3-one 3-(dimethylammonium chloride)propylene acetal



glycolipid with acetal linkage

Fig. 4.
Acid-sensitive lipids with acetal linkage.

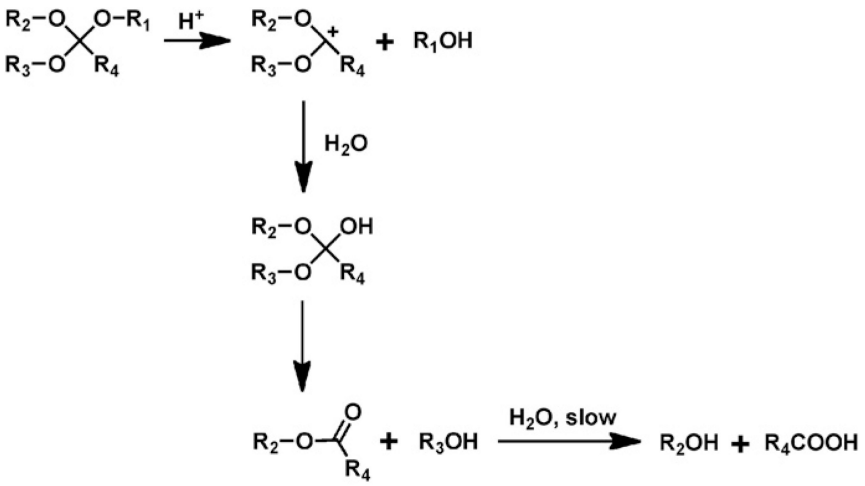


Fig. 5.
General mechanism of acid-catalyzed hydrolysis of ortho esters.

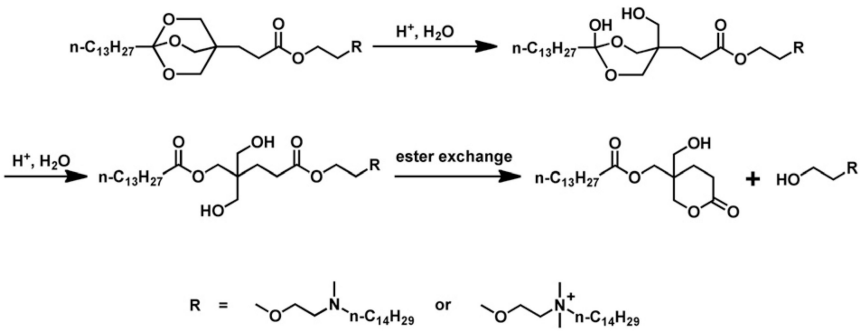


Fig. 6.
Hydrolysis of ortho esters derived from 3,5,8-trioxabicyclo[2.2.2]octane.

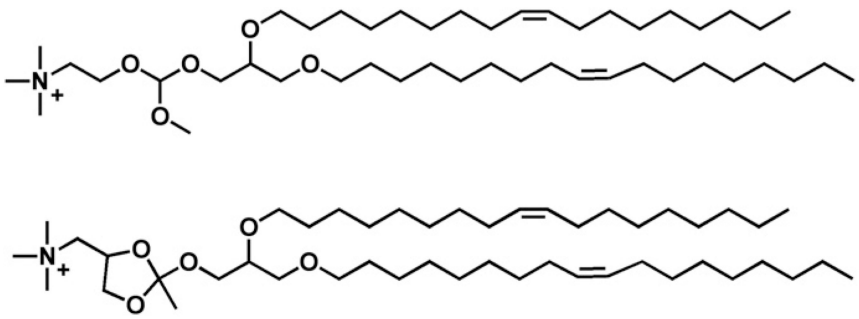


Fig. 7.
Unsaturated cationic ortho esters lipids.

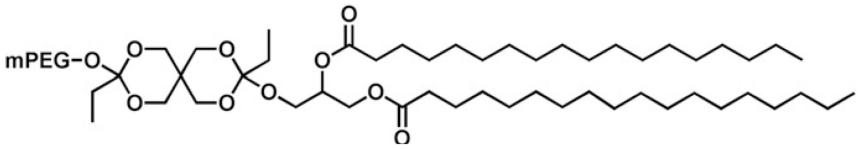


Fig. 8.
A diortho ester conjugate of PEG and distearoyl glycerol.

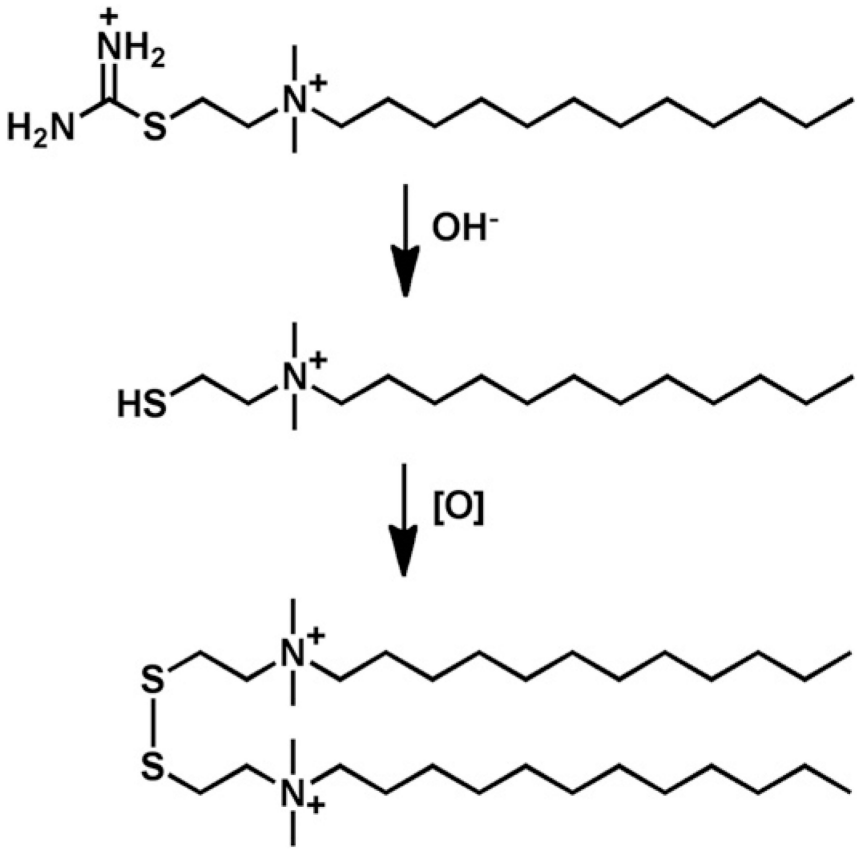


Fig. 9.
Base-sensitive cationic isothiuronium surfactants.

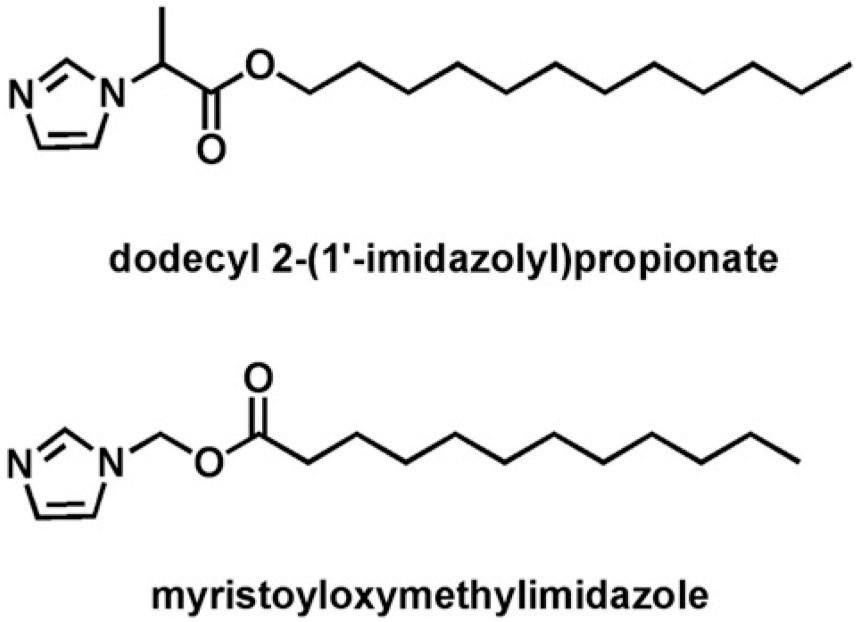


Fig. 10.
Imidazole-derived pH-sensitive surfactants.

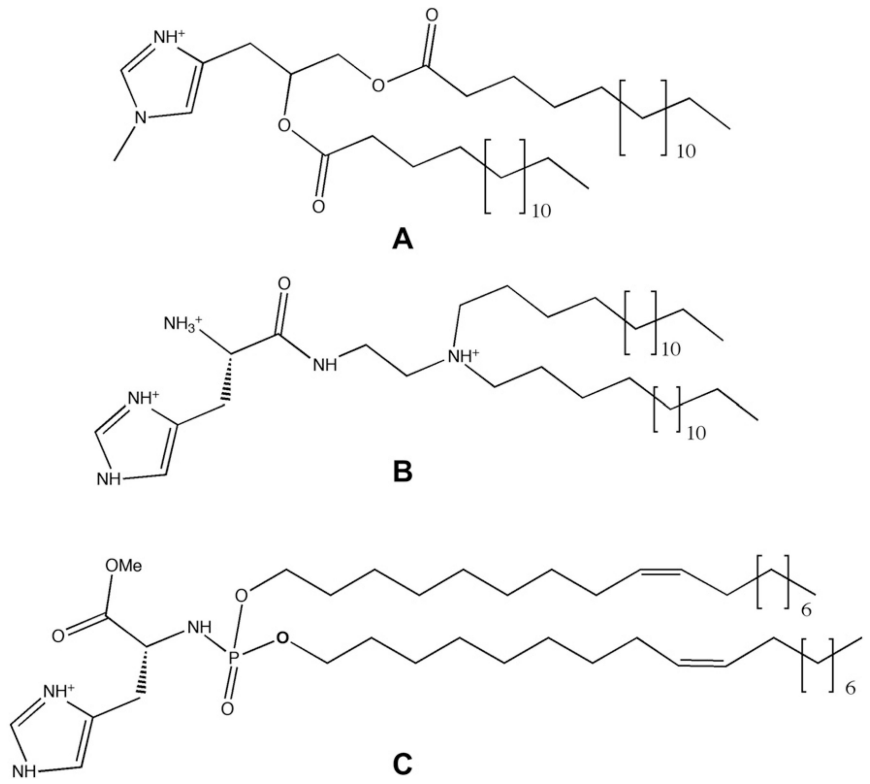


Fig. 11.
Three examples of imidazole and histidine pH lipids used for gene delivery.

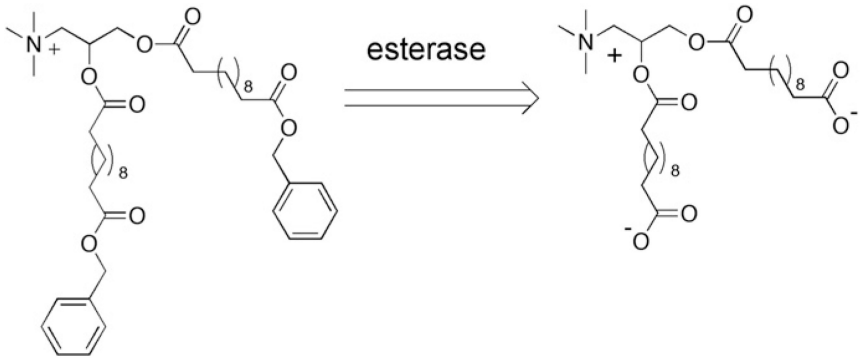


Fig. 12.
Charge-reversal amphiphile.

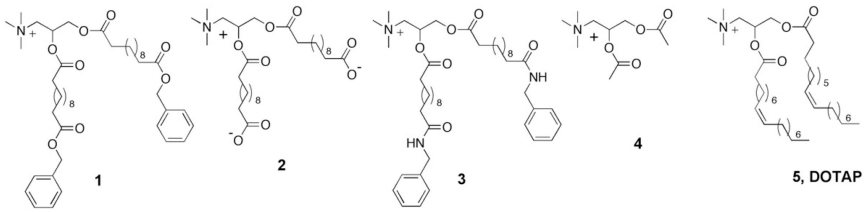


Fig. 13.
Amphiphiles studied.

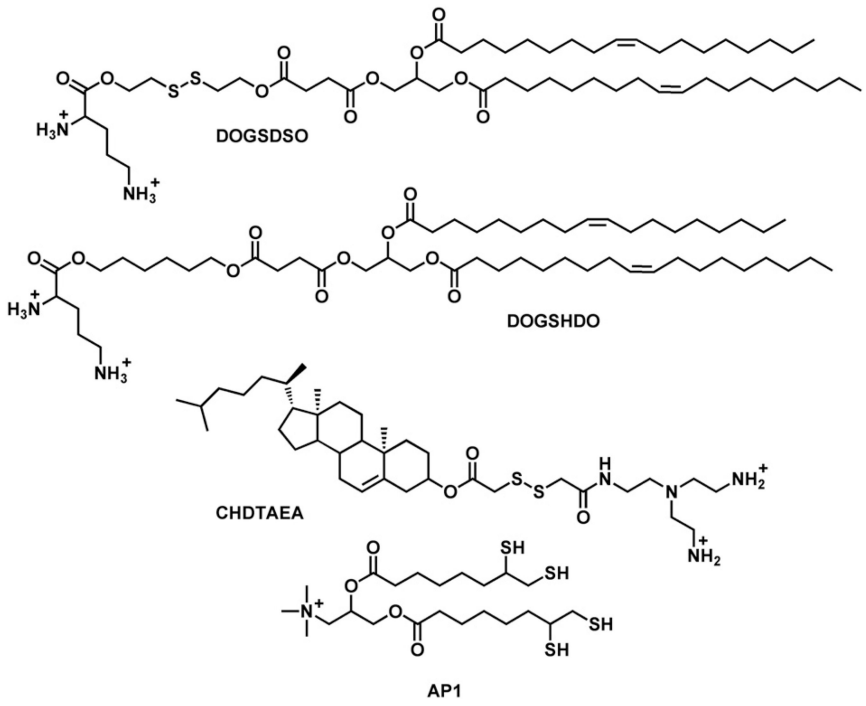


Fig. 14.
Redox-responsive lipids DOGSDSO, CHDTAEA and AP1.

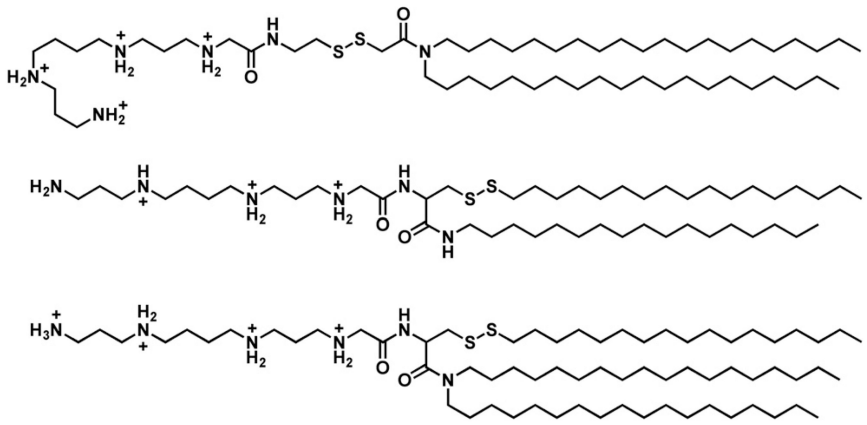


Fig. 15.
Cationic lipids with dithiol groups at different positions.

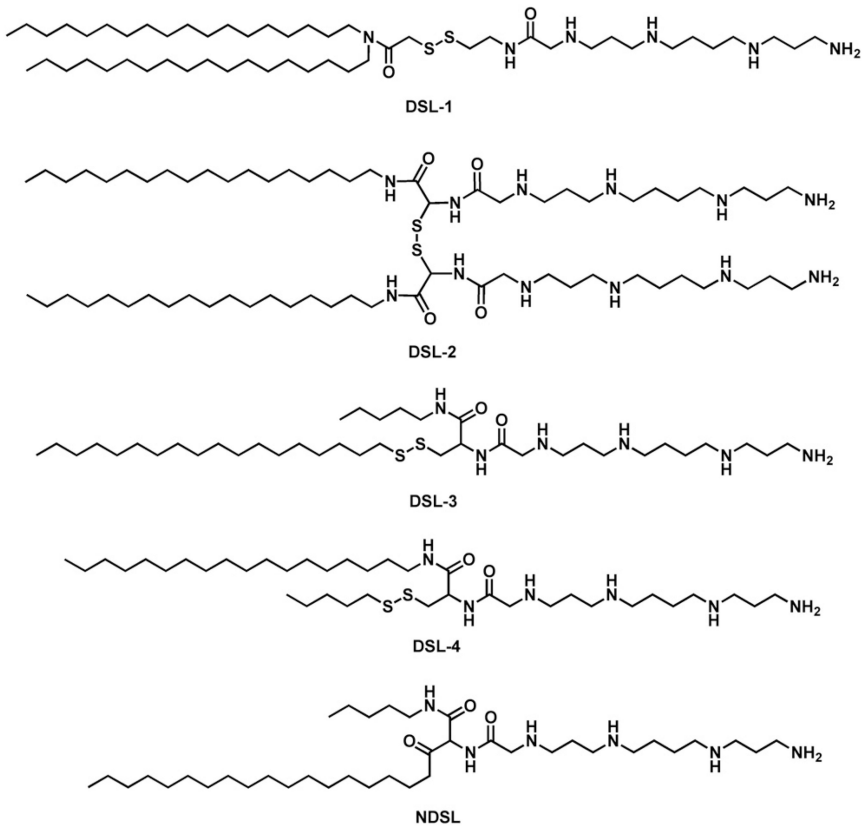


Fig. 16.
DSL series reducible lipids.

Table 1

Current lipid-mediated gene therapy clinical trials.

Indication	Gene	Clinical phase	Lipid vector	ClinicalTrials.gov Identifier:
advanced head and neck cancer	EGFR antisense DNA	Phase I	DC-chol	NCT00009841
cystic fibrosis	pGT-1 gene	Phase I	DMRIE/DOPE	NCT00004471
non-small-cell lung cancer	Fus1 gene	Phase I	DOTAP:Chol	NCT00059605
advanced pancreatic cancer	Bik gene	Phase I	cholesterol-based liposome	NCT00968604
cystic fibrosis	CFTR gene	Phase I/II	lipid 67 and helper lipids	NCT00789867
solid tumor cancers	M2 subunit of ribonucleotide reductase (R2) siRNA	Phase I	stabilized nanoparticles	NCT00689065
advanced solid tumors with liver involvement	VEGF and KSP siRNA	Phase I	lipid nanoparticles	NCT00882180
advanced solid cancer	PKN3 siRNA	Phase I	AtuFECT01/DPhyPE/DSPE-PEG	NCT00938574
respiratory syncytial virus infections	nucleocapsid N gene siRNA	Phase II	lipid nanoparticles	NCT01065935
transthyretin mediated amyloidosis	transthyretin gene siRNA	Phase I	lipid nanoparticles	NCT01148953

Source: www.clinicaltrials.gov