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Review

Polymers for DNA Delivery

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Abstract: Nucleic acid delivery has many applications in basic science, biotechnology, agriculture, and medicine. One of the main applications is DNA or RNA delivery for gene therapy purposes. Gene therapy, an approach for treatment or prevention of diseases associated with defective gene expression, involves the insertion of a therapeutic gene into cells, followed by expression and production of the required proteins. This approach enables replacement of damaged genes or expression inhibition of undesired genes. Following two decades of research, there are two major methods for delivery of genes. The first method, considered the dominant approach, utilizes viral vectors and is generally an efficient tool of transfection. Attempts, however, to resolve drawbacks related with viral vectors (e.g., high risk of mutagenicity, immunogenicity, low production yield, limited gene size, etc.), led to the development of an alternative method, which makes use of non-viral vectors. This review describes non-viral gene delivery vectors, termed "self-assembled" systems, and are based on cationic molecules, which form spontaneous complexes with negatively charged nucleic acids. It introduces the most important cationic polymers used for gene delivery. A transition from in vitro to in vivo gene delivery is also presented, with an emphasis on the obstacles to achieve successful transfection in vivo.

Keywords: Gene-delivery, cationic-polymers, dextran–spermine.

Introduction

The discovery of DNA and its function in the orchestration of life has presented us with unimaginable and endless possibilities in numerous fields of science. This has led to diverse functional applications. Agriculture is one of the fields that has strongly benefited from the possibilities of genetic engineering, which has resulted in a more sustainable food supply at lower costs of production [1]. For example, a gene that codes for herbicide resistance has been put in several crops such as soybeans, maize, wheat, rape, fodder/sugar beet, and chicory. This manipulation allows the crop to be sprayed with herbicides without killing the crop. In addition, genes coding for proteins toxic to insects have been isolated and transferred to crop plants such as maize, potato, and cotton. If insects eat the leaves they are killed. A widely used application is preventing the expression of a softening enzyme in tomatoes, allowing them to be ripened on the plant while reaching the supermarket sufficiently firm for sale. Other applications in agriculture include modified starch content in potatoes; altered lignin content in the poplar tree, modified oil content, reduced pod shatter, fungal tolerance, and male sterility in the rape plant; and Arctic turbot antifreeze protein genes in strawberries.

Gene technology has not only facilitated production in agriculture; pharmaceutical products are produced more efficiently as well [2]. This way large-scale production of substances with complicated structures such as insulin, human growth hormone and factor VIII has been realized. The production systems consist of genetically altered bacteria, yeast, and animal cell lines, and also of whole animals, such as goats that secrete antithrombin III or monoclonal antibodies in their milk.

In fundamental research in several fields of science, gene manipulation serves a wholly different, but equally important, role. By activating, silencing, introducing, or knocking out genes both in vitro and in vivo, attempts are made to understand how organisms work, what causes malfunctions, and what are the possibilities for interventions. For example, introduction of certain genes to tissues or organisms can provide models for a wide range of diseases or behavioral aspects.

Gene delivery for therapeutic application currently involves two strategies: corrective or cytotoxic gene therapy. The former includes correction of genetic defects in target cells. This strategy is exploited for the treatment of diseases with single gene disorders (e.g., severe combined immunodeficiency syndromes, cystic fibrosis, hemophilia, sickle cell anemia, β-thalassemia, muscular dystrophy) and malignant tumors, including ovarian carcinoma [3]. The latter strategy includes destruction of target cells using a cytotoxic pathway. This strategy is used for treatment of uterine leiomyomata and of malignant tumors, including ovarian, breast, and endometrial carcinoma [3].

The administration of genes for therapeutic purposes can be done in vivo or ex vivo. In vivo administration encompasses direct administration of the gene or vector into the patient or into the target organ, and potentially can be applied to any cell. The ex vivo administration includes harvesting and cultivation of cells from patients, with in vitro gene transfer and reintroduction of transfected cells. The potential target cells for this administration include lymphocytes, bone marrow cells, umbilical cord blood stem cells, hepatocytes, tumor cells, and skin fibroblasts [3]. The main challenge of gene therapy, whether ex vivo or in vivo, is still the delivery of DNA to target cells accompanied with a high level of desired gene expression.

Gene delivery systems

The feasibility of widespread gene therapy application depends upon continuing development of suitable methods for gene delivery. In fact, the greatest obstacle in the field revolves around the engineering of appropriate vectors [4]. Current vectors battle a lack of specified cell targeting in vivo, inefficient long-term expression, and low transfection rates [5]. Additional considerations include the capacity of the vector to package genes of sufficient size and the vector's immunogenicity. Therefore, an ideal gene therapy delivery system would be injectable, targetable to specific sites in vivo, regulatable, able to maintain long-term gene expression, and be nonimmunogenic [4]. Currently used delivery systems can be divided into viral and nonviral vectors. Viral and nonviral vectors present specific advantages and disadvantages [3].

Viral delivery systems

As the concept of gene therapy expanded, various viral vectors were explored as potential vehicles due to their natural ability to transport their genomic DNA into the nucleus of the host cell while evading degradation by lysosomes. Creating a vector involves producing a recombinant virus lacking replication but maintaining its ability to infect cells. Current gene therapy research tests viruses such as retroviruses, lentiviruses, adenovirus, and adeno-associated virus for their vector capability. Among the most studied viruses, retroviruses were the first viral vectors to be explored [6]. The retrovirus gains cell entry through the interaction between viral envelope glycoproteins and cell surface receptors [7]. Once internalized, viral RNA is transformed to DNA with the resulting complementary DNA (cDNA) undergoing incorporation into the host genome as a provirus. Molecular virology studies have revealed a potential solution to the major disadvantage of traditional retrovirus vectors, namely, the need for active cell proliferation. Lentivirus vectors exhibit stable in vivo delivery of genes into nondividing cells [8–10]. Adenovirus presents an alternative method to introduce genetic material into cells. This common DNA virus produces a wide range of human infection, including acute febrile upper respiratory infections, keratoconjunctivitis, and hemorrhagic cystitis [7]. Host cells infected with wild-type adenovirus undergo cell lysis, resulting in viral load release. Therefore adenovirus is most effectively applied in cytotoxic gene therapy. In hopes of circumventing the drawbacks of adenovirus while taking advantage of its attractive features, investigators have explored the use of adeno-associated virus, which were efficient in both differentiated and nondifferentiated types, as well as nondividing cells and hematopoietic cells.

Nonviral delivery systems

The nonviral systems developed for gene delivery include: (1) *Direct DNA delivery* — Direct DNA delivery entails direct injection of DNA (naked DNA) into the target organ. This method was useful in delivery of DNA into skeletal muscle [11–12], liver [13], heart muscle [14], and tumors [15]. However, naked DNA goes through rapid degradation upon systemic administration. Other delivery modes include particle bombardment with DNA-coated metal pellets shot into the cell [16] and electroporation [17–18]. Direct DNA delivery has also led to the development of the DNA tumor vaccines [19]; (2) *Encapsulated DNA* — Encapsulation of DNA into neutral and anionic liposomes has

also been proposed as nonviral delivery system. An anionic liposome cannot externally bind negatively charged DNA, which must be encapsulated, allowing cell-specific targeting. Therefore the DNA size to be encapsulated is limited [20]; (3) *Artificial chromosomes* — The most recently described nonviral vector is the mammalian artificial chromosome. After diagnosis of a genetic defect, introduction of the mammalian artificial chromosome into a subset of blastocysts by microinjection would allow a sufficient population of cells to express the transgene to eliminate the genetic deficiency [21]; (4) *Self-assembled complexes* — The self-assembled complex is the most commonly employed nonviral strategy. This strategy includes primarily the use of complexes consisting of DNA and cationic lipids or cationic polymers (which are the basis for this thesis), and is discussed in details below.

Self-assembled nonviral vectors

Cationic polymers and cationic lipids are capable of spontaneously forming complexes with DNA after removal of small counterions from both cationic carriers and DNA (a thermodynamically favored step, which drives and stabilizes complex formation) [22–23].

Cationic polymers

Polymers can be specifically designed for the proposed application by choosing appropriate molecular weights, coupling of cell- or tissue-specific targeting moieties or performing other modifications that confer upon them specific physiological or physicochemical properties. A scale-up to the production of large quantities is rather easy as well. Cationic polymers used for nucleic acid delivery acquire their charge from primary, secondary, tertiary, and/or quaternary amino groups, which are capable of forming electrostatic complexes with DNA under physiologic conditions. For example poly-L-(lysine) (PLL) and its derivatives [24–25] contain primary amines; polyamidoamine (PAMAM) starburst dendrimers [26–27] have primary and tertiary amines; branched polyethylene-imines (PEI) possess primary, secondary, and tertiary amino groups, while linear PEI have mostly secondary amines [28–32]. Diethylaminoethyl (DEAE) dextrans [33] possess tertiary amines; chitosan and its derivatives [34–35] have primary or modified quaternary amino groups; and poly(dimethyl-aminoethyl methacrylates) [36] contain tertiary amino groups. Some of the most studied polycations used for gene delivery (i.e., PEI, PLL, and PAMAM, Figure 1), as well as our recently developed polysaccharide-oligoamine conjugates are discussed below.

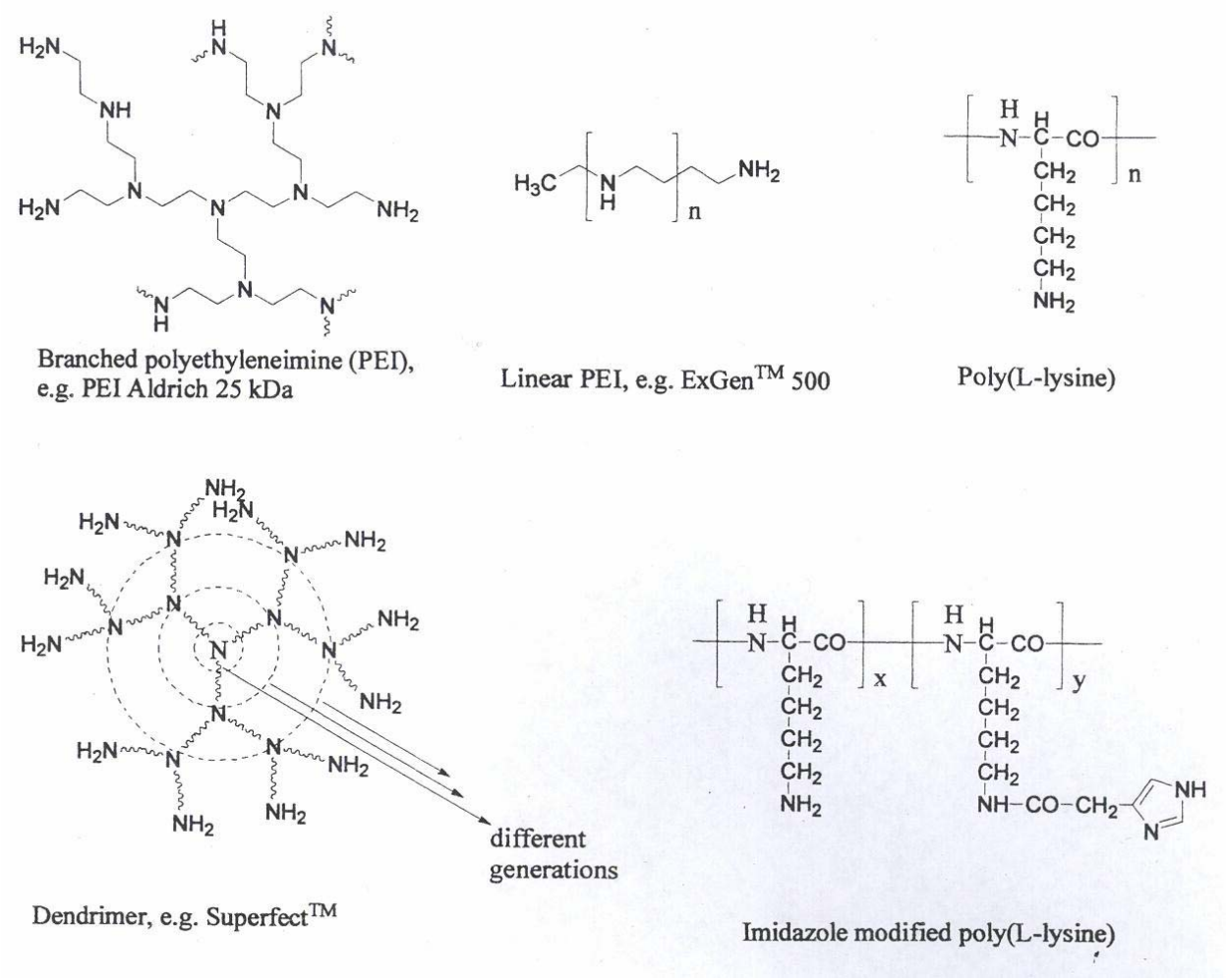
PEI

PEIs were first introduced by Behr in 1995 [37], and have become one of the gold standards of nonviral gene delivery. Highly branched PEI [e.g., 25-kDa (Aldrich) and 800-kDa (Fluka)] and linear PEI are most frequently used [28–30], and were found to be capable of transfecting cells efficiently in vitro as well as in vivo. PEIs offer a significantly more efficient transfection and protection against nuclease degradation than other polycations, e.g., PLL, possibly due to their higher charge density and more efficient complexation. The high amount of positive charges, however, results in a rather high toxicity of PEI polymers. The toxicity and the fact that these polymers are not biodegradable are limiting factors, especially for its in vivo use [28, 31]. The high density of primary, secondary, and

tertiary amino groups exhibiting protonation only on every third or fourth nitrogen at pH 7, confers significant buffering capacity to the polymers over a wide pH range. This property, known as the "proton sponge effect" [37] (see below) is probably one of the most important factors explaining the high transfection efficiencies obtained with these polymers.

Not only the PEIs molecular weight, but also the degree of branching plays an important role for biological properties of complexes with nucleic acids. Linear PEIs [38] have been synthesized and investigated [39], and it was demonstrated that linear PEI 22 kDa, e.g., ExGen™ 500 (Euromedex, France), displays excellent transfection efficiency [31, 39–40]. Linear PEI has recently been reported to mediate a cell-cycle-independent nuclear entry of plasmid DNA [41]. This finding is of particular importance in the therapy of slowly-dividing tissues.

Figure 1 Structures of cationic polymers commonly used for gene delivery (Adapted from [47])



PLL

PLL was one of the first polymers used in nonviral gene delivery, and a large variety of polymers with different molecular weights have been utilized in physicochemical and biological experiments [42]. Due to its peptide structure, PLL is biodegradable, a property that makes it especially suitable for in vivo use; however the polymer exhibits modest to high toxicity. Its polyplexes are taken up into

cells as efficiently as PEI complexes, however transfection efficiencies remain several orders of magnitude lower. A potential reason for this is the lack of amino groups with a pK_a between 5 and 7, thus allowing no endosomolysis and low levels of transgene expression [43]. The inclusion of targeting moieties or co-application of endosomolytic agents like chloroquine [44] or fusogenic peptides [45] may improve reporter gene expression. In addition, attachment of histidine or other imidazole-containing structures to PLL (i.e., pK_a around 6, thus possessing a buffering capacity in the endolysosomal pH range) showed a significant enhancement of reporter gene expression compared to unmodified PLL [24–25, 46].

Dendrimers

Dendrimers are spherical, highly branched polymers prepared either by divergent (starting from a central core molecule) or convergent (starting with what will become the periphery of the molecule, building inwards) synthesis strategies. The degree of branching is expressed in the generation of the dendrimer. The 6-generation Starburst™ PAMAM dendrimers are the most commonly used dendrimers for nonviral nucleic acid delivery, either in intact (Polyfect®) or fractured (Superfect®) form. Similar to PEI, the structures of these polymers show high densities of amines in the periphery of the molecule. These outer amines enable efficient condensation of nucleic acids, leaving the inner amine functions available for a proton sponge effect during endolysosomal acidification, thus enabling more efficient endosomal escape. Concerning 6-generation PAMAM dendrimers, the fractured dendrimers show significantly enhanced (>50-fold) levels of reporter gene expression compared to the intact polymer. The reason for this finding is still unclear, however an increased flexibility of the polymer with a better ability to complex DNA might play a crucial role [26].

Polysaccharide-oligoamine based conjugates

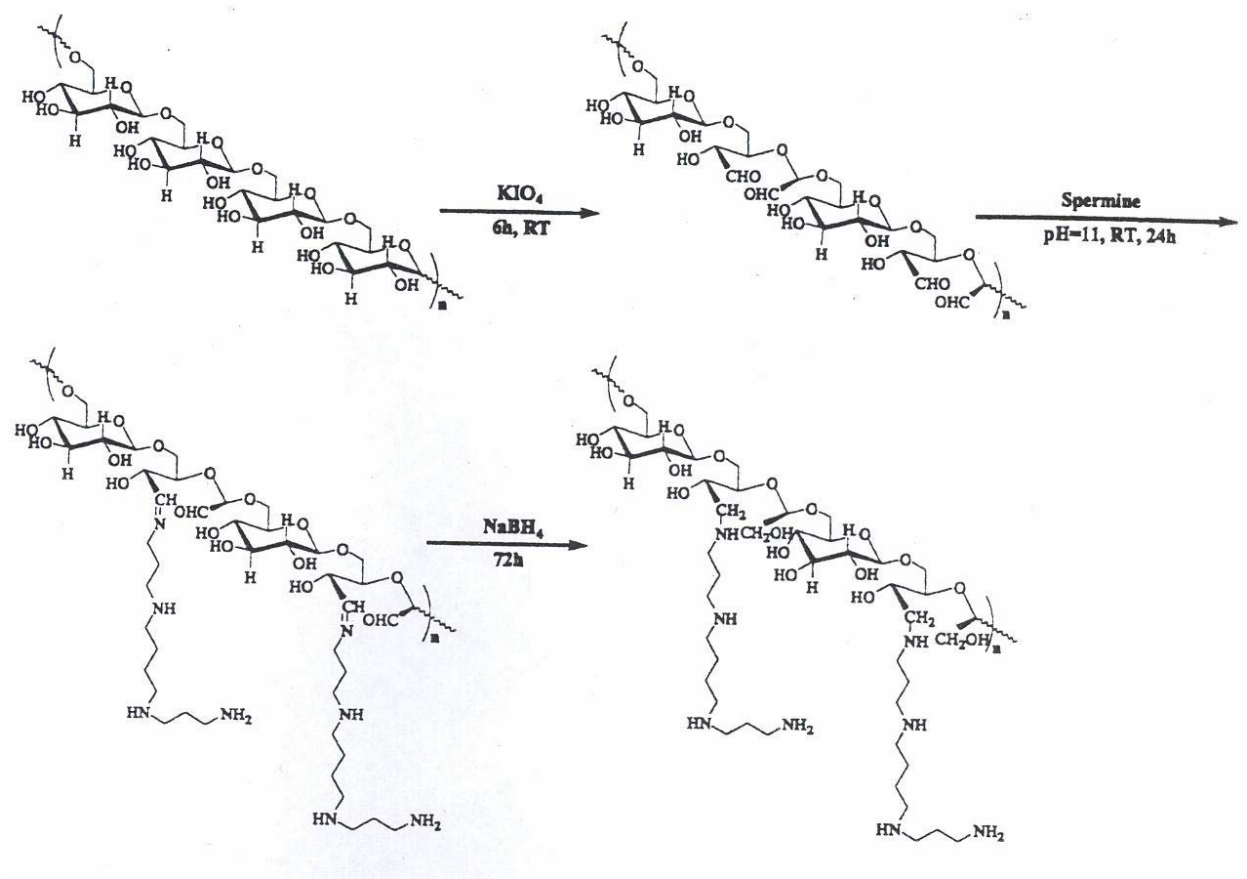
In recent publications [48–51], we reported on the development of a new type of biodegradable, water-soluble polycation based on grafted oligoamine residues on natural polysaccharides. The grafting concept where side chain oligomers are attached to either a linear or branched hydrophilic polysaccharide backbone (Figure 2), was thought to allow two/three dimensional interaction with an anionic DNA chains. The oligoamine distribution along a polymer chain at a certain distance from each other (e.g., every one, two, three, or four saccharide units) was thought to provide controlled charge density (which allows finding optimum physicochemical properties and transfection efficiency).

Synthesis

The polysaccharides used for the synthesis were highly branched arabinogalactan (AG, 19 kDa), pullulan (Pu, ~50 kDa), and dextrans (D) with an average molecular weight ranging between 9.3 and 500 kDa. The representative polysaccharide was oxidized by potassium periodate (KIO_4/sugar units 1:1, 1:3, and 1:5, mole ratio) in water, resulting in polyaldehydes. Oxidation at 1:1 mole ratio of D and Pu yielded a 50–60% oxidation of the saccharide units, with a marked decrease in molecular weight. The lower ratio of KIO_4 oxidized the two polymers to a lesser extent, with minimum chain scission. Oxidation of AG under similar conditions yielded less oxidation with minimal change in the molecular

weight. The difference in the degree of oxidation between the polymers was found to depend on the structure of the polysaccharide. D and Pu are linear polysaccharides with glucose units connected by 1,6- and 1,4-glycoside bonds, respectively. These bonds allow oxidation of the polymer backbone, resulting in high dialdehyde formation with some chain scission. AG saccharide units, on the other hand, are connected by 1,3-glycoside bonds, which are stable to oxidation; thus, oxidation takes place only in the branched chains.

Figure 2 Synthesis of polysaccharide–oligoamine conjugates



More than 300 different polycations were prepared starting from AG, Pu, and D with various molecular weights. Each polysaccharide was oxidized at three oxidation degrees as described above, and the obtained polyaldehydes were allowed to react with the corresponding oligoamine under basic conditions, resulting in the imine conjugates. Reduction of the imine conjugates with $NaBH_4$ yielded the more stable amine conjugates (Figure 2). The oligoamines used for conjugation were alkanediamine of two amine groups (e.g., ethane-, propane, butane-, hexane-, and octanediamine), triethylene glycol diamine, diethylenetriamine, dimethylethylene- and dimethylpropylenediamine, polyethyleneimine oligomer of an average molecular weight of 600 Da, the naturally-occurring spermine and spermidine, and synthetic spermine analogues with 4 amine groups (e.g., *N,N*-bis(3-aminopropyl)-1,3-propanediamine; *N,N*-bis(3-aminopropyl)-1,2-ethanediamine; and *N,N*-bis(2-aminoethyl)-1,3-propanediamine). Most of conjugates were soluble in water and water mixtures with alcohols, DMF, DMSO, and tetrahydrofuran. The synthetic conjugates were purified by extensive dialysis to obtain pure polymers uncontaminated with salt and low molecular weight oligoamines, as

judged by GPC. The %N content varied between 2.5 and 12% and was dependent mainly on the aldehyde content of the starting polysaccharide. The average molecular weights of conjugates based on D and Pu were 8,000±2000 Da. The drastic decrease in the molecular weight of these conjugates compared to the starting polysaccharides is explained by the extensive aminolysis of the glycoside linkages during conjugation [52]. The average molecular weights of cationic conjugates based on AG resulted in similar or even higher molecular weights compared to starting AG. The reason for this behavior results from on the structure of the polysaccharide. AG is a highly branched polysaccharide (~45% branching) where the polymer backbone does not contain geminal hydroxyl groups and the saccharide units are connected by 1,3-glycosdie linkages and thus is less sensitive to oxidation with KIO_4. Therefore, the only glycoside units of AG that are available to oxidation are the branched units, which are connected together by 1,4- and 1,6-glycoside linkages. Unlike D and Pu where the aminolysis takes place on the polymer backbone during the conjugation process and therefore in substantial chain scissions, aminolysis in AG takes place only in the branched chains without major effect on the molecular weight of the polymer.

In vitro transfection

Polysaccharide and substituted oligoamine — Transfection efficiencies of the polycations were performed applying NIH-3T3 cells and pLNC-luciferase as the marker gene. DOTAP/Chol (1/1) and Transfast™ (Promega®) cationic lipids, as well as calcium phosphate (Sigma) were used as positive controls. Each single polymer was tested at a wide range of charge ratios (–/+, phosphate/nitrogen) starting from 1 to 0.05. When simple diamines were applied as the grafting oligoamines (i.e., ethylene, propylene, butane, hexane and octane diamines), no transfection was obtained in all grafted polysaccharides. PEI600, *N,N*-dimethyl ethylenediamine and *N,N*-dimethy lpropylenediamine also resulted with negligible transfection efficiencies in all grafted polysaccharides. Only the dextran-spermine based conjugate was found to efficiently transfect cells in culture in similar transfection efficiency to the positive controls. Replacement of spermine with spermidine as the grafted oligoamine resulted in a drastic decrease in the transfection efficiency. On the contrary of spermine, spermine analogues (*N,N*-bis(3-aminopropyl)-1,3-propanediamine; *N,N*-bis(2-aminoethyl)-1,3-propanediamine; and *N,N*-bis(3-aminopropyl)-1,2-ethanediamine) when applied as the grafted oligoamines resulted with low to negligible transfection efficiency [48].

Type of conjugate (amine vs. imine) — Unlike amine based conjugates (reduced) of dextran-spermine, imine-based conjugates (un-reduced) showed no transfection activity in all conjugates. This behavior could be explained by the fact that only spermine of four amino groups conjugated to dextran is effective. Thus, the imine derivatives were inactive because the side chain is of three cationic groups as the imine bond is not cationic. Another explanation to this behavior is the fact that imine bonds are labile under acidic conditions and could be easily breakdown inside the endosome where the pH drops below 5.5 [49].

Molecular weight of polysaccharide and conjugate — High transfection efficiencies were obtained with conjugates having an average molecular weights of 8,000±2000 Da, whereas conjugates having relatively high molecular weights (>20,000 Da) synthesized from high molecular weights dextrans

resulted in low to negligible transfection efficiencies. Dextran–spermine polycations of low molecular weights are assumed to form relatively stable complexes rather than those obtained with high molecular weight conjugates [49].

Cationic lipids

Although several attempts had been made to use neutral or negatively charged liposomes for gene delivery, the limited efficiency of plasmid–DNA encapsulation and consequently the low levels of transfection encouraged investigators to focus on cationic liposomes [53]. Cationic liposomes were able to complex and condense DNA [54], and were proposed as efficient carriers for the intracellular delivery of DNA [55].

Structure size and morphology of cationic liposomes

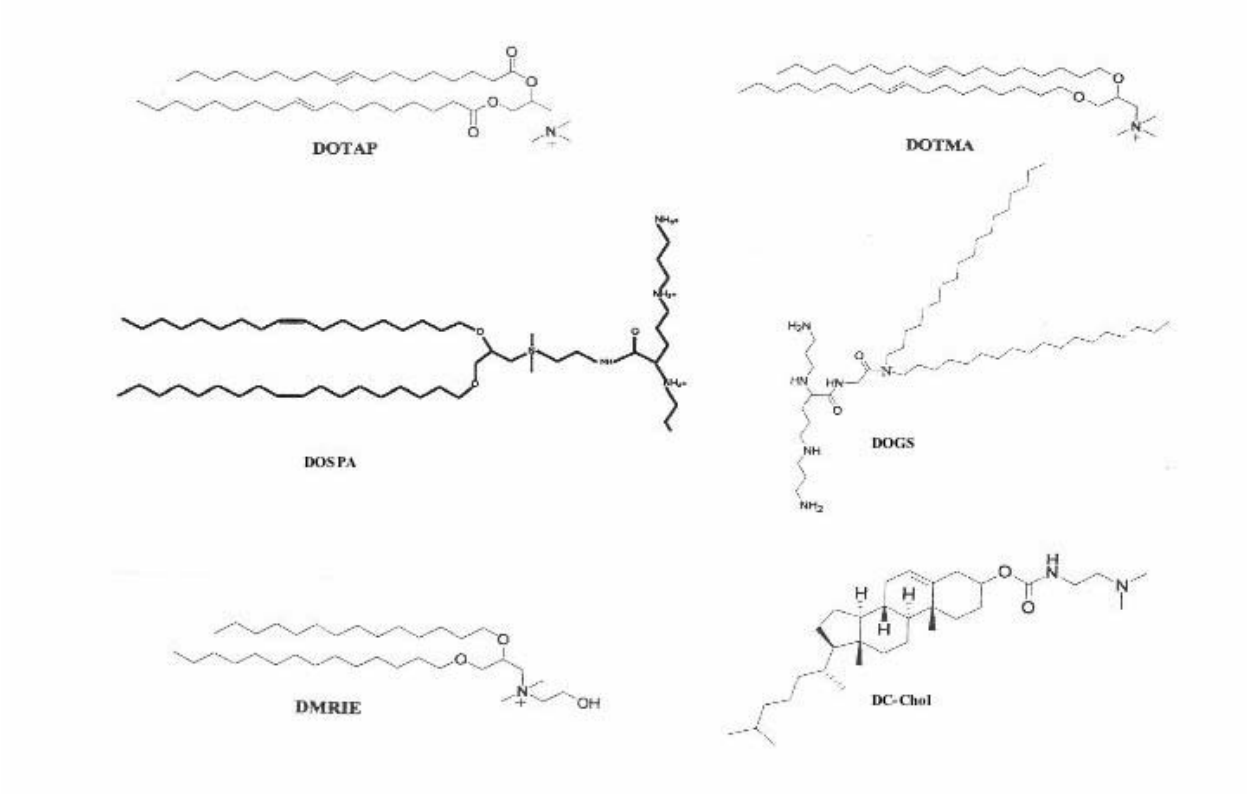
Over the last few years an enormous amount of work has been devoted to the development of novel formulations of cationic liposomes, namely through the synthesis of different cationic lipids with low toxicity and exhibiting different abilities to mediate gene transfer [56–62]. The first cationic lipid for transfection, DOTMA, was introduced by Felgner in 1987 [55]. In order to gain biodegradability and reduced toxicity [63] DOTMA ether bond was replaced with an ester bond to obtain DOTAP [64]. A typical cationic amphiphile (Figure 3) generally comprises three important elements: cationic headgroup, hydrophobic anchor, and linker. The positively charged headgroup is necessary for binding and complexation of nucleic acid phosphate groups. While the function of the hydrophobic part is less clear, it probably assists in assembling the lipids into a polycationic scaffold as well as in facilitating absorptive endocytosis and/or fusion with cell membranes. All cationic lipids are classified according to number of positive charges, nature of linker bond, and nature of hydrophobic anchor. For example, DOTAP represents the group of monocationic quaternary surfactants, in which the amine-based cationic headgroup is connected through a linker to two hydrocarbon tails. In general, reports indicated that the myristoyl (C14) chain is optimal, followed by oleoyl (C18:1) [56, 65] and hydrocarbon chain asymmetry is unfavorable for transfection [65]. A direct correlation between the nature of the linker group of the cationic lipids and their potential cytotoxicity was also demonstrated. Lipids with stable ether linkages (e.g. DOTMA, DMRIE) are more toxic than those containing labile ester linkages (e.g. DOTAP) [56, 66–67].

It has been demonstrated that for a given liposome composition, unsized heterogeneous vesicles (UHVs, 300 to 700 nm) [68], when complexed with DNA mediate higher transfection activity than lipoplexes prepared from small or large unilamellar cationic liposomes [20 nm (SUVs) or 100 nm (LUVs)] [56, 69–71]. However, the resulting lipoplexes (from SUVs LUVs or UHVs) do not differ significantly in their size (ranging from 300 to over 2000 nm, depending on the composition of the medium used in their preparation) or in the extent of their cell association and uptake.

The ability of DOTAP to form liposomes by itself, without addition of neutral lipids, is similar to that of other monocationic lipids like DOTMA and DMRIE. The geometry of lipids could be described by the ratio between cross section of the hydrophobic part and the polar headgroup area (i.e., packing parameter, which allows one to roughly predict whether a given lipid molecule favors inverted hexagonal, lamellar (bilayer), or a micellar assembly [72]). DNA addition to cationic liposomes result

in either lamellar or inverted hexagonal phase structure. The lamellar is a condensed and globular structure, consisting of DNA monolayers, characterized by a uniform interhelical spacing, which are sandwiched between cationic lipid bilayers [73]; while the inverted hexagonal phase structure consists of DNA coated by cationic lipid monolayers arranged on a two-dimensional hexagonal lattice [74–75]. DOSPA and DOGS, which are multivalent cationic lipids, form micellar rather than vesicular structures [76] and exhibit a higher efficacy in condensing DNA than monovalent lipids (e.g. DOTMA, DOTAP, DC-Chol, DMRIE). This property, however, does not necessarily lead to a higher transfection efficiency, since the intracellular dissociation of DNA from the complexes is expected to be more difficult [77].

Figure 3 Structure of cationic lipids commonly used for gene delivery (Adapted from [78])



Helper-lipids

The choice of the helper lipid has major impact on the structure and the activity of lipoplexes. A helper lipid can improve the ability of cationic liposomes to transfect cells. In vitro studies show that liposomes composed of an equimolar mixture of DOPE and cationic lipids (e.g. DOTMA, DOTAP) can mediate higher levels of transfection than those containing only the cationic lipid or a different helper lipid like DOPC [70, 79–81]. This fact has been attributed to the ability of DOPE tendency to undergo a transition from a bilayer to an hexagonal configuration under acidic pH, which may facilitate fusion with or destabilization of target membranes, in particular endosomal membranes [23, 56, 71, 74]. It was suggested that DOPE can also play a role in facilitating the disassembling of the lipoplexes after their internalization and escape of DNA from endocytotic vesicles [82–83]. This was based on the assumption that due to salt bridge between the quaternary amine and PE phosphate the amine group of PE can interact with DNA phosphate groups, thus leading to weakening of the binding

reaction between cationic lipids and DNA [23, 82]. Cholesterol when used as a helper lipid form more stable but less efficient complexes than those containing DOPE in vitro. However, cholesterol-containing lipoplexes have shown higher biological activity compared to lipoplexes with DOPE when these complexes were utilized in vivo [58, 68–69, 84–87]. The significant transfection activity achieved was attributed to an improved cell binding and uptake of the lipoplexes promoted by the presence of cholesterol [88] and/or better stability of the lipoplex in serum [68].

Transfection of cells in culture

Generally the in vitro transfection efficiency of a polyplexes or lipoplexes depends on a large variety of factors including complexes preparation (e.g., concentration of the carrier and DNA solutions, order of mixing (addition of DNA to carrier or vice versa), ionic strength of the solutions, dose, charge ratio, and speed of mixing), the cells (e.g., cell type, confluency of cells, cell physiological state, degree of cellular metabolism, rate of division and cell cycle) and other factors such as composition of incubation medium, and time of incubation. In addition, inside the cell the nucleic acid has to be carried across several cellular barriers in order to reach its desired site of action and to display the desired therapeutic effect. Therefore, optimal formulation should consider each of these factors and their influence on complex properties, cellular uptake, toxicity and transfection efficiency. The steps in transfection of cells in culture as well as cellular barriers are discussed below.

Uptake of cationic complexes

The first obstacle to overcome is traversing the cellular membrane. This membrane is composed of a lipid bilayer and various integral proteins, it acts as a gatekeeper, which selectively screening all foreign matter entering the cell. Cell surfaces are negatively charged, due to their content of glycoproteins, proteoglycans and glycerolphosphates [89]. The predominant routes of entry for polyplexes, is adsorptive endocytosis following the clathrin coated pit mechanism [90–92] or fluid phase endocytosis [93]. Lipoplex were shown to bind the cell surface through association with the negatively charged extracellular proteoglycans as well [94–95]. The extent of binding both lipoplex and polyplex to the cell surface and high complexes internalisation does not necessarily translate into a similar enhancement of transgene expression [96–100]. Upon uptake, lipoplexes undergo fusion with cellular membranes. Friend et al. [101] demonstrated signs of fusion of DOTMA/DOPE lipoplexes with cell plasma membrane, but the exact role of this process in transfection is still unclear [102, 103]. Some publications suggest a phagosomal, rather than an endosomal route of uptake for lipoplex, based on the inhibition of cell association by cytochalasin B [104] (a inhibitor of phagocytosis) and on the size considerations [i.e., lipoplex size is often bigger than the average endosome size (100 nm)]. However, recent data suggest that the clathrin-mediated pathway of lipoplex uptake (i.e., endosomal uptake) is involved [103]. It is possible, however, that the mechanism of lipoplex uptake (phagocytosis vs. endocytosis) depends on the size of the particles used in the study, the specific lipid composition, and the cell type. In general view, the uptake of complexes is a “must” but is not considered a major obstacle on the way to achieve transfection.

Several strategies were proposed to improve cellular uptake: (1) *Cationization of complexes* — Increasing the positive surface charge of complexes [105] result in a higher affinity to negatively

charged membrane constituents and subsequently to a higher rate of uptake (at the same time, an increase in positive surface charges may also result in increased toxicity); (2) *The use of viral protein transduction domains (PTD)* — These proteins are capable of mediating the entry of large biomolecules directly into the cytoplasm without the use of endocytotic mechanisms. Some even promote transport across the nuclear envelope [106–107]; (3) *Targeting* — Complexes coupled to targeting moieties may be taken up by receptor mediated endocytosis (e.g., fibronectin [108] or kistrin [109] that bind to integrins [110–113], transferrin that bind to transferrin receptor [83, 114–116], saccharide ligands that bind to asialoglycoprotein receptor (ASGPr) [117–122], antibodies that bind to their target structures [123–125], growth factors that bind to growth factor receptors [126–127] etc.)

Escape from the endosome

Following internalization, the next step is the release of the complexes from the endocytotic compartments into the cytoplasm. Whatever the mechanism of complex uptake (e.g., phagocytosis, adsorptive or fluid phase endocytosis) they end up in the acidic organelles (i.e., the complexes follow the scheme of the endolysosomal pathway, leading from the early to late endosomes, and ultimately ending in the lysosomal compartment). The lysosomal vesicles fuse and assemble in the perinuclear region [93]. Here the majority of complexes remain without significant changes in distribution patterns [43]. In order to be effective, complexes or at least their nucleic acid component must escape from this route since the lysosomal environment, with its aggressive nucleases and acidic pH of approximately 5, eventually destroys the potential efficacy of the entrapped complexes. This endosomal escape seems to be one of the key steps controlling productive delivery of nucleic acids to the cytoplasm (RNA) or to the nuclei (DNA, ODN).

Two mechanisms were proposed for endosomal escape:

(1) Proton sponge effect — Polymers that exhibit high transfection efficiencies, such as PEI [28], Starburst Dendrimers™ [26], imidazole-containing polymers [24–25, 46, 128] or lipopolyamines such as DOSPA [129–130] exhibit high buffering capacity, in the lysosomal pH range of 5–7 (due to presence of unprotonated secondary and tertiary amines), and high transfection efficiency. The `proton sponge effect' has been proposed [37] as an explanation for the mechanism of such complex release. According to this hypothesis the buffering capacity of the carrier leads to increased influx of protons and chloride ions during endolysosomal acidification, which results in increased osmotic pressure in the vesicle. As a consequence, the passive diffusion of water into the vesicle increases, thus leading to swelling and eventually rupture or leakage of the vesicle. The expansion of the carrier structure, due to repulsion of positive charges may contribute to the vesicle destabilization [25, 43, 131].

(2) Membrane destabilization — Lipids having quaternary amine such as DOTAP do not possess a buffering effect. However, the lipoplex efficiency was partially attributed to a rapid and efficient escape from the acidic lysosomal compartment, or to delayed transfer to lysosomes, thus enabling DNA survival. There is sufficient evidence that lipoplexes are able to destabilize endosomal/lysosomal membranes [101, 132–134] and it was demonstrated that efficient cationic lipid formulations are able to perforate the endosomal membrane, whereas less efficient lipid formulations are not [135]. This process is explained by electrostatic interaction between the oppositely charged cationic lipids of

lipoplex and anionic phospholipids composing intracellular compartments [134] (or by fusogenic potential of lipids such as DOPE [136]) leading to disturbances in the curvature of the vesicles and finally lead to leakage [137] or bursting and release of endosomal contents to the cytoplasm [132]. The role of this phenomenon on transfection efficiency, as well as its correlation with lipoplex structure, is poorly understood. In addition, this mechanism for endosomal escape has been proposed for polyplex as well showing that high generation PAMAM dendrimers and PEI possess a higher membrane destabilizing potential [compared to low generation dendrimers or poly(L-lysine)] [137–138].

Through the cytoplasm

Stability and mobility of naked DNA in the cytoplasm

While ribozymes and antisense oligonucleotides may already be active in the cytosol, plasmid DNA has to be transported into the nucleus, in order to exhibit the desired gene expression. The cytoplasm is a critical place with respect to stability of DNA and RNA, due to the presence of nucleases that reduce their half-life dramatically. Naked plasmid DNA, for example, exhibits a half-life as short as 50–90 min [139–140]. Since the majority of plasmid DNA enters the nucleus during cell division it must remain stable until the next disassembly of the nuclear envelope. Another factor that plays an important role in nucleic acid transit through the cytoplasm is the rate of mobility, which depends on size and spherical structure of the molecule. The mobility of large molecules, such as plasmid DNA, is extremely low in the cytoplasm [141]. Low mobility means a longer trafficking time to the nucleus and thus prolonged exposure to aggressive nucleases. Microinjection studies have shown that the majority of the injected plasmid remains at its site of injection [142]. Therefore the site where plasmid DNA is released into the cytosol is of great importance for efficient nuclear delivery.

Effects of DNA-complexation with cationic carriers

Increased cytosolic mobility — Complexation of DNA with cationic carriers result in DNA condensation (compacted state) [90, 143]. This compaction could lead to increased cytosolic mobility, as compared to naked DNA [144].

Protection of the nucleic acids from cytoplasmic nuclease degradation — Another reason for the higher level of gene expression of complexes (compared to naked DNA) could be the protection of DNA from cytoplasmic nuclease degradation. Several studies have demonstrated excellent stabilization using cationic complexes [145–146], when comparing the stability of naked and complexed DNA/RNA in the presence of DNases or RNases.

Enhancing the permeability for large molecules — It was demonstrated that cationic carriers could interact with f-actin fibres altering the structure of the cytoskeleton and thus enhancing the permeability for large molecules [147–148].

Dissociation of DNA from the complex

The process of DNA dissociation from complexes was attributed to anionic molecules in the cell cytoplasm capable of replacing the anionic DNA [134–149]. In the case of lipoplexes containing DOPE, it was suggested that DOPE may also be involved in helping the DNA dissociation from the lipoplexes due to the ability of its amine group to compete with cationic lipid for DNA phosphate groups, upon lipoplex internalization [23, 82]. It was also suggested that the mechanism for complex dissociation involves components in the endosome or pre-lysosomal membrane [150]. In some cases, the DNA dissociation takes place in the nucleus [149, 151].

Into the nucleus

The final barrier for transfection is the nucleus. The nuclear compartment is surrounded by the nuclear envelope, which consists of a double membrane interrupted by large integral protein structures (the nuclear pore complexes, NPC) [152]. NPCs contain 50–100 functionally distinct proteins [153] (nucleoporins), which are involved in transport processes or form the structure of the NPC. During mitosis in vertebrates these proteins are fragmented into ~12 subcomplexes, which then are reassembled at the end of the telophase to form the structure of the pore complex again [153].

Two paths lead into the nucleus:

(1) During mitosis the nuclear membrane disassembles and, thus, even large molecules, such as plasmids are able to gain access [154].

(2) During interphases, the only way to enter the nucleus is through the NPC. Small molecules (< ~50 kDa, ~10 nm [155] or ions are able to diffuse passively through the NPC. The size and the steric properties of plasmid DNA [156–157] makes its enter via passive diffusion difficult [158]. Therefore, it was found that the passive entry of plasmid DNA becomes less efficient with increasing size [159], and no more than 0.1% of the plasmid copies microinjected into the cytoplasm reached the nucleus [144]. Larger molecules, such as proteins or RNA, require an "identification tag" that is recognized by receptors and, thus enables translocation into and out of the nucleus. The upper size limit for this form of entry is ~26 nm (~8 million Da) [160]. This size limit varies not only between species, but also within the same cell line depending on the confluency or energy status of the cells [160]. The diameter of the substrate seems to be the most important property for passage across the NPC, as no limitation for the length of a substrate was found. These "identification tag" are termed nuclear localization sequences (NLS) if they mediate transport into the nucleus and nuclear export signals (NES) if they enable exit from the nucleus. An NLS is in fact a short amino acid sequence that enables the active transport of proteins or viral DNA into the nucleus [161]. NLS do not conform to a specific consensus sequence, very likely because they interact with different receptors. Several studies have demonstrated that the incorporation of NLS resulted in enhanced nuclear uptake and transgene expression [162–169].

From *in-vitro* to *in-vivo*

Although cationic complexes have proven to be very successful in transfecting cells in tissue culture (and to day most of the cells can be transfected with at least one of the many transfection agents available), it has been well recognized that their *in vitro* efficiency does not correlate with their relatively poor *in vivo* activity [58–59, 62, 68, 84, 88, 170–171]. This low *in vivo* efficacy was attributed to the differences in the biology, functionality and complexity between cell cultures and animal models as well as to the changes in the complexes structure upon their interaction with cells and biological fluids. Ideally, the complex should be delivered exclusively to target tissue where it subsequently taken up and further processed on the cellular level. However, upon *in vivo* administration (especially i.v.) the complexes must first go through the biological milieu (e.g., blood), a process which may comprise several obstacles.

Hurdles at the systemic level

Problems resulting from cationic surface charge — Unmodified cationic complexes exhibit numerous problems when applied systemically. Their cationic surface charge leads to numerous unspecific interactions with negatively-charged cellular blood components, vessel endothelia and plasma proteins (albumin, fibronectin, immunoglobulines, complement factors or fibrinogen) [172]. These interactions lead to very short plasma half-lives [173].

Due to their size and the high number of cationic charges, cationic complexes can activate the complement system [174] in a manner that suggests a correlation between the density of accessible positive surface charges and the extent of complement activation. Activation occurs via attachment of complement components, i.e. factor C3, to the complex surface and eventually leads to complex removal by the reticular endothelial system (RES).

Interactions with plasma proteins play a major role in determining circulation time and cellular uptake. The major component, albumin, is primarily responsible for the rapid clearance of complexes from the bloodstream [175]. It has been demonstrated that interaction of albumin with complexes leads to the formation of ternary complexes with a reversed surface charge [175–176], resulting in the formation of large aggregates [173, 176]. These associates are removed rapidly from the bloodstream, presumably via phagocytic capture by scavenger receptors of phagocytic liver cells or via accumulation in fine capillary beds.

The administration of cationic complexes having large excess of positive charge was one of the ways used to overcome the inhibitory effect of serum on transfection [177–178]. However, the administration of such complexes leads to aggregate formation with cellular blood components, especially with erythrocytes [179]. Subsequent obstruction of blood vessels accompanied by undesired consequences, such as pulmonary embolism [172] may be the result. The complex-mediated aggregation of erythrocytes also influences the biodistribution and gene expression patterns of complexes in vivo, resulting in enhanced accumulation in the lung, due to a certain sieve effect of the pulmonary capillaries.

The endothelial barrier — One major problem for systemic gene delivery is the transfer of agents beyond the endothelial barrier. Extravasation of complexes is highly dependent on their size and the

permeability of the endothelia at specific sites. In most tissues the structure of the endothelia is tight. Only organs and tissues with an irregular fenestration, such as the liver, spleen, bone marrow and certain tumors, have endothelia with large meshes. Thus, in most tissues the access of complexes to parenchymal cells is denied.

Biodistribution and gene expression after i.v. application

Organ distribution of the cationic complexes after i.v. injection usually shows a high accumulation in the lung, possibly due to aggregate formation with blood cells or plasma proteins and subsequent filtration in fine lung capillaries [68, 176]. Due to the low stability of such aggregates, complexes are often released into the circulation again, leading to a secondary redistribution with high concentrations found in Kupffer cells of the liver. The endothelial tissue of other organs and tissues, for example spleen, kidney and especially endothelia close to the site of injection, accumulate significant levels of complexes as well [180].

The highest levels of gene expression after i.v. administration are usually found in the lung, not only due to enhanced deposition, but also due to a more efficient gene expression in this organ [68, 181]. Although the lung capillaries possess a tight endothelia, reporter gene expression after i.v. application has been measured not only in endothelial, but also in interstitial cells [182–183]. Some studies have even discovered a rapid crossing of the endothelial barrier by polyplexes [183], although the mechanism of this transport has yet to be elucidated. It has been suggested that at sites where the vasculature is fragile, as in the alveoli, small complexes may be able to pass the endothelial barrier, due to vascular leakage [184]. Other mechanisms, such as transport via a form of transcytosis or the existence of transport systems for polyamines, have also been postulated. Support for the latter theory has been shown in studies demonstrating that polyamines, such as putrescine, spermine or spermidine, are taken up into arterial endothelial cells via specific polyamine carrier systems [185–186]. Overall the lung represents an attractive organ for nonviral gene delivery for the treatment of, for example, cystic fibrosis or lung cancer.

Steric stabilization of complexes

The pattern of organ distribution showing initial deposition in the lung and subsequent rapid uptake predominantly into Kupffer cells of the liver is of limited value for therapeutic application. Hence, strategies have been developed to change this biodistribution pattern and to prolong circulation, thereby enabling the targeting of specific tissues.

Steric stabilization involves the attachment of highly hydrated polymers to complexes, thus shielding positive surface charges and creating a steric barrier against aggregation with, for example, albumin, complement factors or cellular components in the bloodstream.

Several approaches have been tested for their ability to shield the cationic surface charge of polyplexes such as the use of: (1) *polyethylenglycol (PEG)* [84, 86, 172, 180, 187–189] — The in vivo application of PEGylated complexes displayed a slightly prolonged circulation time compared to unmodified complexes. However, the circulation half-lives were still rather short. A decrease in gene expression in the lung and a lower initial toxicity were observed, when compared to unmodified complexes, most likely due to decreased interactions with blood constituents and, therefore, a lower

rate of deposition in lung capillaries via filtration. Significant gene expression was also detected close to the site of injection, suggesting a still significant rate of nonspecific electrostatic interactions; (2) *transferrin* — Transferrin has been demonstrated to effectively shield the surface charges of polyplexes [190]. When incorporated at appropriate densities it leads to a significant decrease of nonspecific interactions with erythrocytes. In vivo studies using charge neutral transferrin-PEI/DNA complexes displayed the accumulation of DNA primarily in the liver (Kupffer cells) and tumor tissues (showing 100–500-fold higher reporter gene expression in tumor tissues compared to other major organs, including the lung) [190]; (3) *Poly(N-(2-hydroxypropyl)methacrylamide) (pHPMA) and multivalent HPMA (lateral stabilization)* — pHPMA polymers have displayed versatile properties as polymeric carriers for a large variety of drugs with excellent biocompatibility [191]. The formation of electrostatic complexes consisting of poly(L-lysine) and plasmid DNA with the subsequent attachment of pHPMA to uncomplexed ε-aminogroups led to decreased interactions with albumin and reduced association with macrophages in vitro [192]. In vivo experiments, however, did not display a prolonged circulation time in this study and liver uptake was even higher than for uncoated complexes. It was suggested that lateral stabilization by multivalent HPMA copolymers is needed [193]; (4) *crosslinking of primary amines* — Crosslinking of primary amines of poly(L-lysine)/DNA complexes via disulfide bonds has shown promising results [194]. In circulation those complexes are stable thus offering efficient stabilization of the DNA, however when taken up into cells disulfide bonds are reductively cleaved and DNA is released. Such crosslinked complexes showed 10-fold increased plasma circulation after i.v. administration compared to unmodified complexes with similar levels of reporter gene expression in vitro.

Local application

If a particular tissue or organ needs to be targeted in nonviral gene or oligonucleotide therapy, local application may in many cases represent a more promising approach, since several barriers of systemic application can be avoided. This approach has been utilized for a variety of cases, including tumors [39, 195–197], kidney [198], lung [199], brain [32, 200–202], heart [203–204], skin [205], muscle [206], and arterial blood vessels [207–208]. However, local administration has to overcome several obstacles as well. For example, in the lungs, the first obstacle of gene delivery to and across the epithelial cells of the lung is a mucus layer secreted by goblet cells, which creates a mechanical barrier against access to the plasma membrane of the epithelial cells. Furthermore, the epithelium itself hinders the uptake of particulate structures, due to its dense structure with actin strengthened apical surfaces and characteristic tight junctions between cells inhibiting intercellular transport. Additionally, countless alveolar macrophages constantly patrol the lung removing particles from the deeper airway via phagocytosis. However, the instillation of poly- or lipoplexes in the respiratory airways was shown to lead to significant gene expression levels [209]. Another method that has been used for lipo- and polyplexes [210–211] is aerosol delivery [212].

Toxicity

As mentioned above, nonviral vectors have the advantage over viral vectors in being less immunogenic. However, immune activation is triggered by systemic and local administration of both

lipoplexes and polyplexes [213–215]. The release of inflammatory cytokines is partially due to the bacterial origin of the plasmid DNA used, which is rich in CpG sequence that stimulate the innate immune system [213]. A more efficient plasmid delivery system invariably induces unspecific immune responses. The effect however also seems to be dependent on the cationic carrier and the route of administration, as was shown for both PEI-DNA polyplexes and BCTG:DOPE-DNA lipoplexes [216]. Furthermore, infiltration of tissue by T cells and NK cells was also seen following administration of cationic liposomes without DNA [217]. Additional to these immunogenic responses, intravenous injection of cationic lipoplexes is often accompanied by a dose dependent toxicity, such as a drop in circulating lymphocytes and an increase of serum levels of liver enzymes [218], and visible hepatic necrosis.

Conclusions

To summarize, nonviral gene delivery still represents a highly promising research area that has great potential for future gene therapy. Remarkable efforts have been made to optimize gene delivery systems in vitro; in vivo application, however, may require different vector features. To achieve the goal of gene therapy numerous hurdles, such as biodistribution to first-pass organs, rapid clearance of complexes, lack of tissue-targeting, toxicity, nonspecific interactions, etc. have to be surmounted.

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