siRNA Transfection with Dendritic Core-Shell Nanocarriers

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1. Introduction

1.1 General Aspects

For more than 30 years, researchers have been working to alleviate diseases through gene therapy,[1-2] but the most important milestone in the evolution of gene therapy came already in 1953 when the molecular structure of the DNA was discovered by Watson and Crick.[3]

Since the emergence of techniques and the first experiments for cloning of mammalian genes in the late 1970s and early 1980s, which are commonly seen as the precursors of gene therapy, this field started rapidly growing, resulting in the first human gene therapy trial in 1989 (Figure 1).[4] The goal of the gene therapy is application dependent. The therapy can be used to override the influence of a malfunction gene through the targeted insertion of a therapeutic gene, to prevent a disease through vaccination, and to inhibit gene expression (gene silencing).[5-6] The inhibition of gene expression can take place either at the transcriptional or post-transcriptional level. Transcriptional gene repression is the result of chemical modifications of the histone, creating an environment of heterochromatin around a gene that makes it inaccessible to transcriptional machinery,[7] whereas post-transcriptional gene silencing is mostly induced by small interference RNA (siRNA).[8] SiRNA was first discovered in 1998 by Fire and Mello and already in 2007 the U.S. Food and Drug Administration (FDA) approved a Quark Pharmaceuticals Inc ("Quark") Investigational New Drug application (IND) for siRNA therapeutic for the treatment of age-related macular degeneration (AMD). Furthermore it is an alternative method to traditional chemotherapy used in treating cancer.[9]

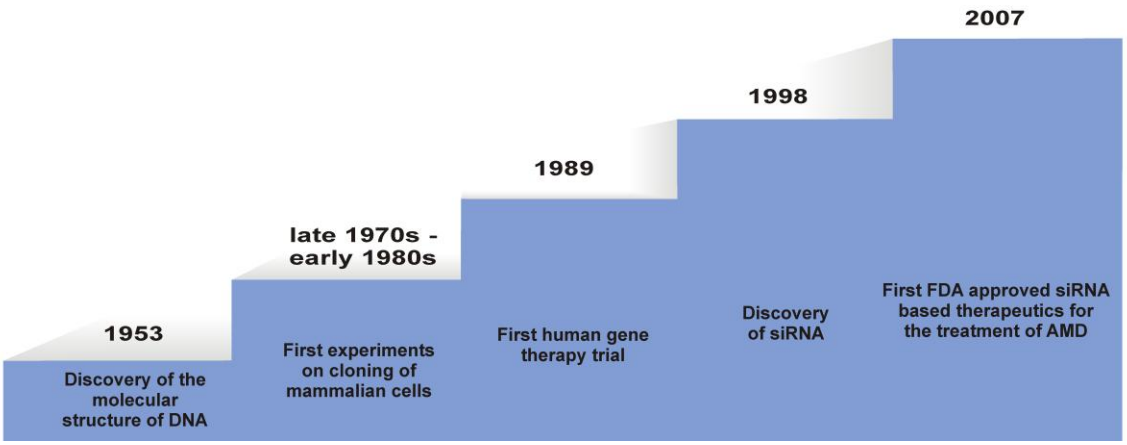


Figure 1. Development of gene therapy from DNA discovery to first RNA therapeutics.

The therapy results in either a cure for the disease or a slowdown in the progression of the disease.[10-12] The first clinical trial of gene therapy which started in 1990 used a retroviral-mediated transfer from the adenosine deaminase (ADA) gene into the T cells (T lymphocytes, a group of white blood cells) of two children suffering severe combined immunodeficiency (SCID). The disease is characterized by a genetic defect in the purine catabolic enzyme adenosine deaminase, which causes large amounts of deoxyadenosine convert to the toxic compound deoxyadenosine triphosphate in T cells, thus disabling the immune system.[13-14] Even though the gene treatment ended after two years, ADA gene expression in T cells still persisted.[13]

The potential scope of gene therapy is enormous. Application of gene therapy spans from ordinary classical genetic diseases with a single defective gene to acquired genetic and multifactorial genetic disorders such as cystic fibrosis, sickle cell anemia, SCID, and Parkinson's disease.[15-18] The main advantage of gene therapy is the potential elimination of the disease state instead of alleviating the symptoms of the disease, as conventional medicines do. Figure 2 indicates the wide range of diseases for which clinical studies are ongoing.[19]

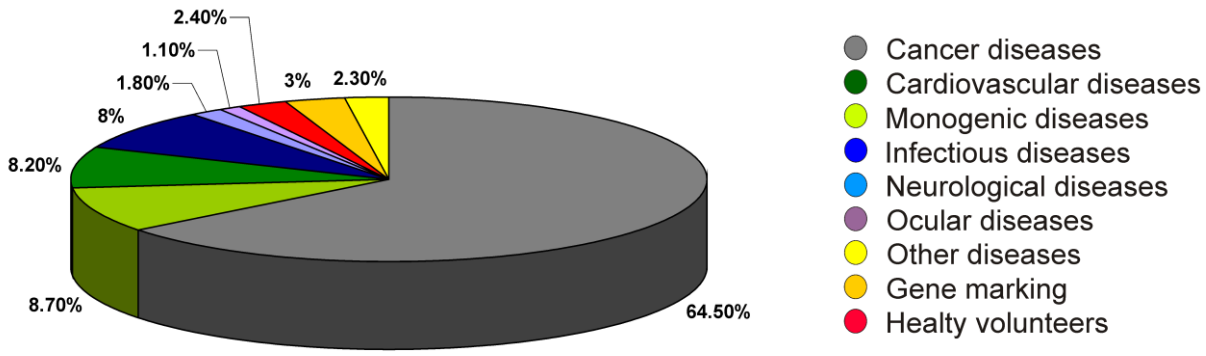


Figure 2. Clinical targets for gene therapy. Adapted with permission of ref. [19-20]. Copyright (2007) John Wiley and Sons.

In principle, all cells in the human body contain genes, making them potential targets for gene therapy. However, these cells can be classified into two types: somatic cells (essentially, non-reproductive) and cells of the germ line.[21-22] Somatic cell gene therapy involves the genetic modification of any cell in a patient's body, whereas germline gene therapy inserts genes into reproductive cells. Since gene therapy in germline cells affect not only the individual cells that develop from it, but also their offspring and successive generations, gene therapy on germline cells is forbidden.[22-23]

The successful application of gene therapy is still a great challenge in ongoing research, since the most difficult obstacles to overcome have been the inability to transfer the

appropriate gene to a target, prolonged gene expression, and to obtain low toxicity.[24-25] Gene transfer of a nucleic acid to somatic cells can take place either in vivo or ex vivo and the process by which genes are transferred into cultured mammalian cells is called transfection.[26] In vivo therapy is accomplished by transfer of genetic materials directly to the patient, whereas in the ex vivo approach, the therapeutic genes are removed from the patient's body, genetically modified in vitro, and finally retransplanted (Figure 3).[25, 27-29]

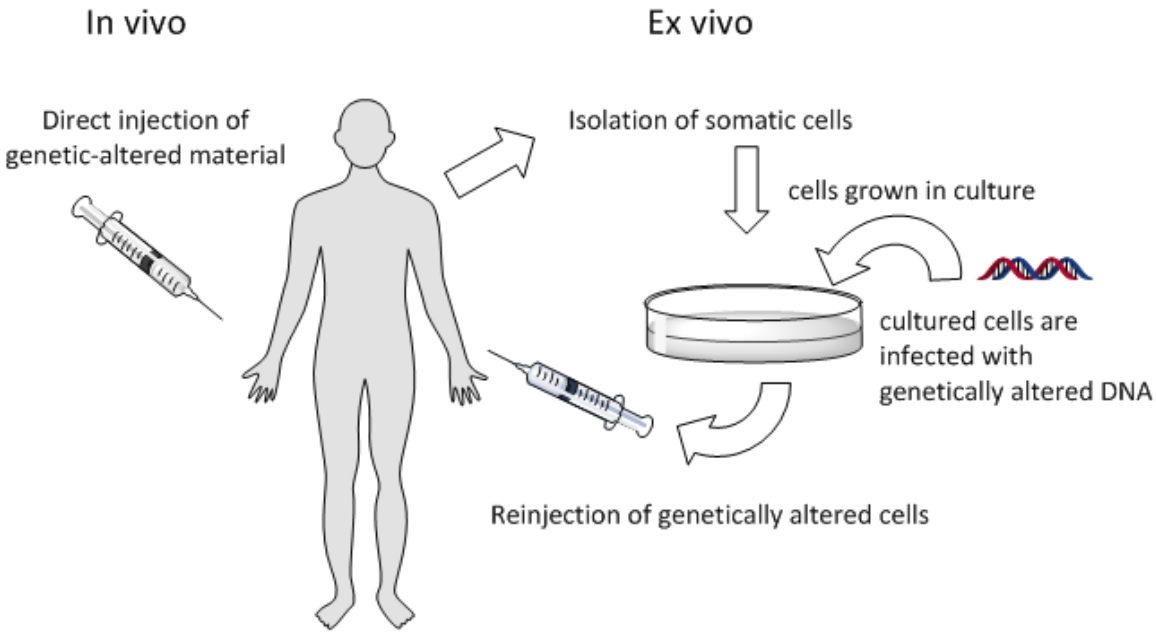


Figure 3. In vivo and ex vivo gene transfer to mammalian somatic cells. Adapted from ref. [30].

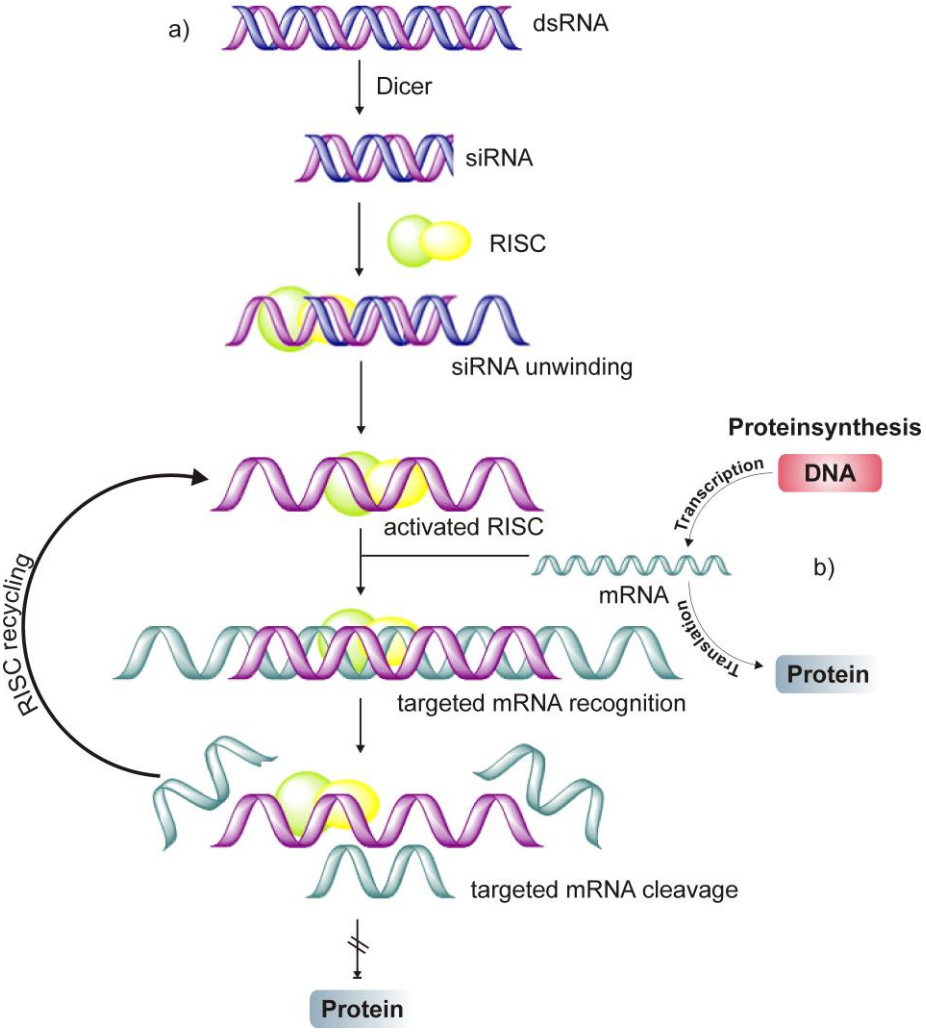
Various types of nucleic acids, e.g., DNA, RNA or siRNA, which achieve different effects at the molecular level, have been delivered into target cells. Since the discovery of RNA interference (RNAi), siRNA mediated gene silencing has attracted considerable research interest.

1.2 RNA Interference

A gene is defined as the region of DNA that provides cells with instructions for making specific proteins.[31] The process by which cells build proteins, called protein biosynthesis, is divided into two steps: (i) transcription, and (ii) translation.[32] These steps are necessary, because the DNA only stores the genetic information but it is not involved in the process by which the information is used.[33] Thus, the genetic information of the DNA has to be transcripted into the messenger RNA (mRNA). Before the information of the DNA can be decoded, a small portion of the DNA double helix must be uncoiled. A single-stranded RNA

is then synthesized that is a complementary copy of one strand of the DNA.[34] The resulting mRNA is able to leave the nucleus and travels through the cytosol to the ribosomes that are made of a small and large subunit which surrounds the mRNA during the translation step where the message is translated into a sequence of amino acids.[33] In general, the mRNA serves as a template for the production of a protein (Scheme 1).[32]

In 1998, Fire and Mello described a new technology, the RNA-mediated interference, which is based on the silencing of specific genes by double-stranded RNA.[35] RNA interference is a mechanism of post-transcriptional gene silencing that has been described in plants, invertebrates, and mammalian cells and is believed to be involved in antiviral defense and regulation of gene expression.[36-38]



Scheme 1. Proposed mechanism of RNAi and simplified synthesis pathway of proteins. Reproduced with permission of ref. [39]. Copyright (2010) The Company of Biologists Ltd.

The trigger for this cellular defense mechanism is dsRNA, which is produced during the replication. Initially, the dsRNAs are cleaved into siRNAs by a ribonuclease known as a

Dicer.[40] The siRNA is double-stranded, and the length usually varies between 21-23 nucleotides. The siRNAs gets incorporated into ribonuleotide protein components called RNA-induced silencing complex (RISC) and by unwinding the duplex, triggered through Argonaute 2, the catalytic core within RISC, the complex becomes activated RISCs.[39, 41] The antisense strand of sequence-specific siRNA binds by base pairing interactions to complementary mRNA molecules and triggers the cleavage of mRNAs (Scheme 1).[35, 41-42] The bound mRNA is cleaved and sequence-specific degradation of mRNA results in gene silencing.[42] The RISC remains still activated after degradation of one mRNA strand and can therefore move on to destroy additional mRNA targets, which results in continuous gene silencing. Depending on the type of cell, the therapeutic effect can last for 3-7 days in dividing cells and even up to several weeks in non-dividing cells.[41] To achieve a persistent effect and to keep a certain therapeutic threshold, repeated administration is necessary, since siRNA can eventually degrade within the cell.[41] The required length is short enough to ensure a low probability that mRNAs other than the desired target will be destroyed.[42] siRNA with no suitable target appear to remain inert within the cell. In practice siRNA can be synthetically produced, since RNAi can be tailored to almost any gene to study the function of genes by knocking out their expression without actually destroying the genetic material.[42]

Since its discovery, siRNA has been widely used to identify gene functions and holds great potential in providing a new class of therapeutics.[43] The existence of RNAi in human cells and the development of RNAi in cell cultures suggest that this procedure could be used as a powerful tool to silence gene-related disease.[42, 44] Preclinical experiments have already demonstrated that synthetic siRNAs are potent inhibitors of a variety of diseases in vivo,[43, 45] including hypercholesterolaemia,[46-47] liver cirrhosis,[48-50] hepatitis B virus (HBV),[51-54] human papillomavirus,[53, 55] ovarian cancer,[56-57] and bone cancer.[58-60] Since the therapeutic benefit of RNAi inevitably relies on the delivery of synthetic siRNA into cells, and its systemic delivery to target cells is complex, the development of efficient gene delivery systems represents a challenging hurdle in the field of gene therapy.[1, 61-62]

1.3 Gene Delivery Systems

The first and the simplest way to deliver naked siRNA is to intravenously administer it, however it is rapidly degraded by nucleases in the bloodstream as well cleared by renal filtration due to its small size. Moreover, it lacks the ability to penetrate the cellular lipid membranes due to its negative charge, resulting in poor cellular uptake and

pharmacokinetics.[63-65] Therefore, successful application of RNA interference in mammalian cells requires an efficient transfection agent that will enhance their pharmacological properties.[39] The appropriate delivery system should be able to: (i) complex/condense the siRNA, (ii) facilitate targeting to and uptake into the target cells, (iii) protect it from degradation through serum nucleases, and (iv) promote trafficking to the cytoplasm to provide the siRNA in clinically relevant doses.[28, 39, 66-69] However, designing appropriate gene delivery systems must take into consideration a number of factors, namely payload, toxicity and targeted release. An effort to design an efficient gene delivery system should try to overcome the following obstacles:

1. Low uptake across the plasma membrane
2. Slow release of condensed siRNA
3. Limited stability
4. Lack of cell targeting

To accomplish these requirements various strategies have been developed, mainly deriving from chemical and non-chemical methods as illustrated in Figure 4.[64-65]

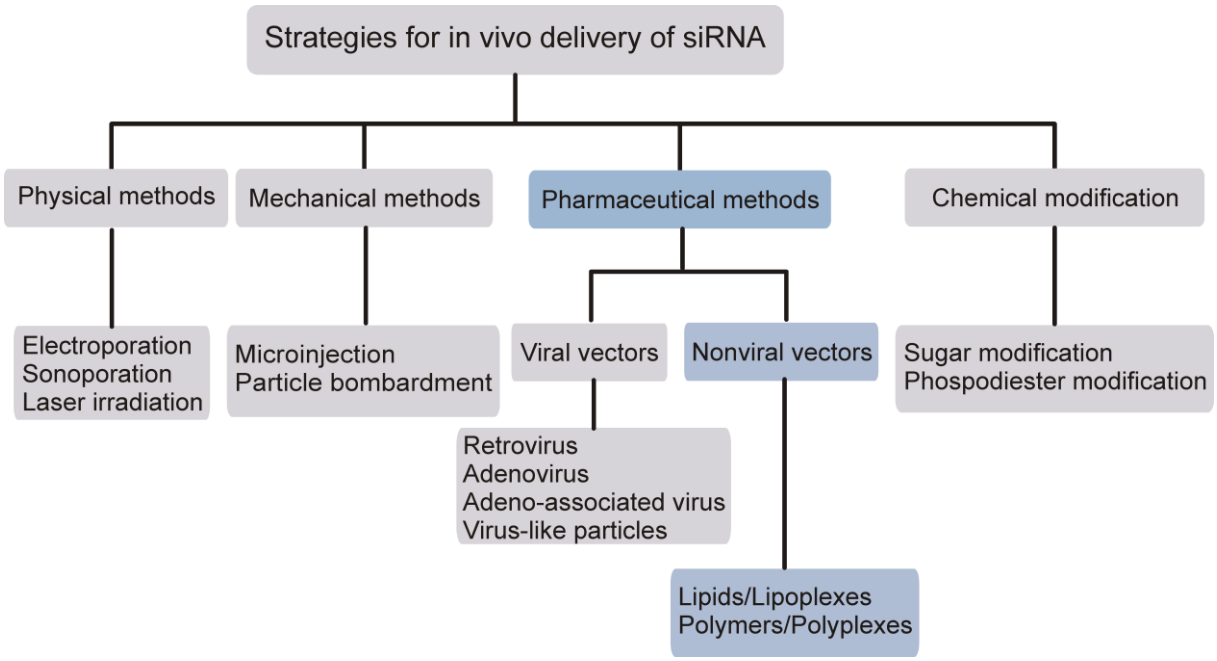


Figure 4. Various strategies to overcome the challenges in delivery of siRNA. Adapted from ref. [65].

1.3.1 Physical Methods

DNA delivery, especially via the non-viral method involves treatment of cells by chemical or physical means, has become a promising alternative to the viral route. The transfer of DNA via the physical route can be achieved in several ways, ranging from electroporation,[70-71] sonoporation,[72] to laser irradiation.[73]

1.3.1.1 Electroporation

Electroporation or electropermeabilization, the application of controlled electric fields to lipid bilayers causes transient and localized destabilization and structural changes such as formation of pores, so-called electropores.[74] These pores permit the uptake of exogenous molecules such as DNA, siRNA, and proteins.[73, 75] This process was first described as a tool to deliver DNA to mammalian cells in 1982 by Neumann et al. since then electroporation has been widely investigated for various cell types.[76] Although electroporation is difficult to apply, successful in vivo gene delivery has been achieved since the early 1990s to skin,[77-78] muscle,[71, 79] liver,[80-81] and solid tumors.[78, 82] Because of the ease of administration, the skin is the most attractive target.[83] In this protocol, a syringe and electrodes (surface, needle electrodes or electroporation catheters) must be inserted in the selected tissue to deliver DNA into a cell (Figure 5).[84] Since only DNA present in the surrounding medium can apparently be internalized, the DNA must be injected before a controlled field is applied. The resultant membrane destabilization allows a significantly larger uptake of DNA.[73] The efficiency of gene transfer by electroporation is highly variable among in the different studies and the use is limited because of the high mortality of cells after high-voltage exposure.[85] Pulse duration, field strength, number of pulses, temperature, DNA concentration, cell number, composition, and cell size need to be optimized and must be determined for each cell type to achieve acceptable results.[86]

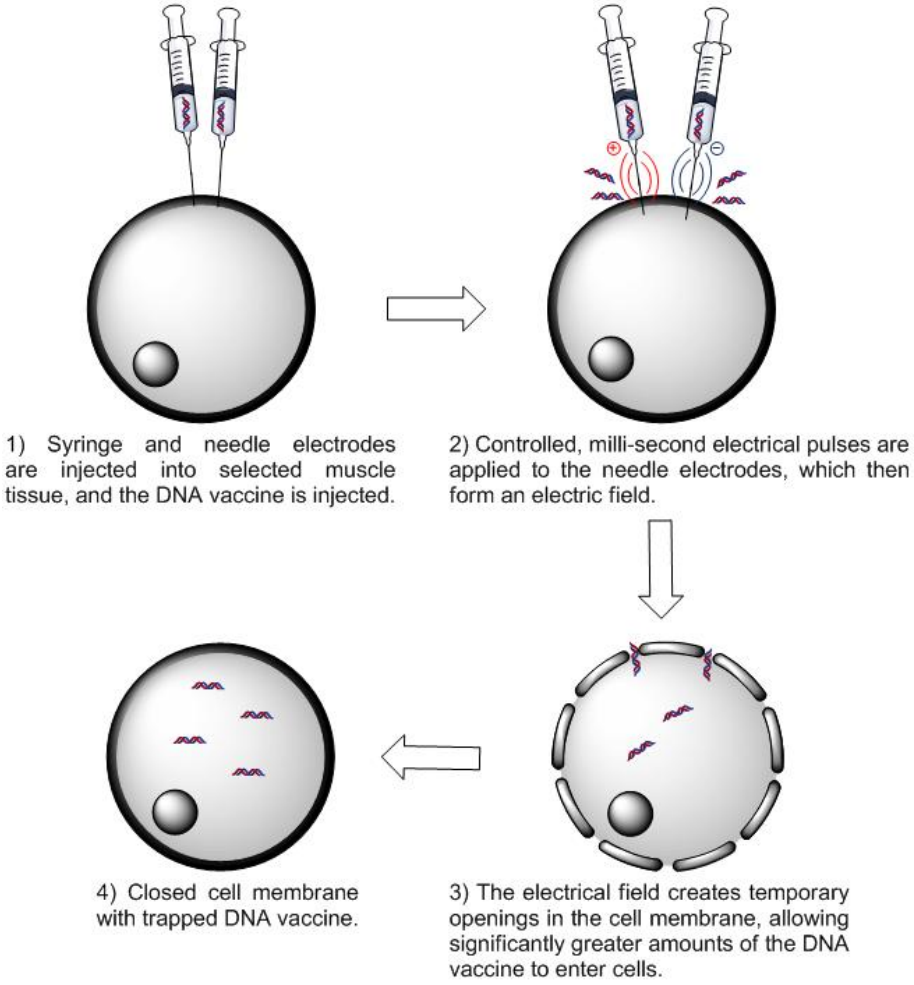


Figure 5. Schematic showing the theoretical explanation how electroporation deliver DNA into the cell. Reproduced with permission from ref. [87]. Copyright (2011) Elsevier.

1.3.1.2 Sonoporation

Sonoporation, the application of ultrasound to lipid bilayers, enhances cell permeability which allows uptake of nucleic acids.[88] In general, only sinusoidal probes are used for a few minutes or less at megahertz frequencies to achieve enough acoustic cavitation to cause bubble implosion and energy release that can permeabilize adjacent cell membranes. Lower frequencies result in lysis of cell, whereas higher frequencies are employed for lithotripsy of kidney and gall bladder stones.[73] An ultrasound contrast agent (Albunex), which consists of elastic and compressible gas-filled microbubbles, was first reported to promote ultrasound-mediated transfection.[89] Nowadays, another contrast agent, known as Optison is preferred, because of its higher efficiency.[90] Like electroporation, sonoporation is supposed to induce structural changes in the cell membrane that causes pores to form. Sonoporation is done with a piezoelectric transducer together with a generator and amplifier. The method can be applied in various tissues, including muscles and solid tumors.[73]

1.3.1.3 Laser Irradiation

Another way to perforate membranes is to apply laser light. Laser irradiation requires a laser source (e.g., neodymium–ytrium–aluminium garnet (Nd:YAG), argon ion, holmium–YAG, titanium sapphire) and a pulse generator.[73] The laser beam is tightly focused by using an objective directed onto the target cell.[91] The perforation allows the nucleic acid present in the surrounding medium to be transferred into the target cell.[92] The transfection efficiency depends on several parameters, including the energy level, the number of pulses, and the pulse duration, even though its limitations are comparable to microinjection, since it can only be applied to a small number of cells.[73]

1.3.2 Mechanical Methods

1.3.2.1 Microinjection

The direct injection of nucleic acids into the nucleus or cytoplasm is microinjection, which is conceptually the simplest approach to gene delivery.[85] Thin glass microcapillary pipettes are used to insert nanoliters of nucleic acid solution into a single cell. A micromanipulator and a microinjector are used to control the movement of the pipette.[93] However, the throughput that every single cell has to be manipulated individually is obviously a major drawback of this method.[94] Microinjection was successfully used in vitro in 1980, when Capecci injected DNA into the cytoplasm and nucleus.[73] The advantage of the direct injection of DNA into the nucleus is that it can bypass cytoplasmic degradation, which results in high gene expression.[95] Even the method is relatively efficient, it can be only used to applications in which individual cell manipulation is desired and possible. Therefore it is not useful for in vivo gene delivery applications at its current technological level.[95]

1.3.2.2 Particle Bombardment

Particle bombardment, which is also often referred to as biolistic particle delivery or a gene gun, can be used to deliver nucleic acid into many cells simultaneously and was originally designed to overcome the inherent difficulty of plant transformations.[73]

In this procedure, gold or tungsten particles coated with DNA are accelerated to a high velocity to penetrate cell membranes and even the nucleus, thereby bypassing the endosomal compartment.[95] The target of a gene gun is most often the skin, where it propels DNA coated

beads into the epidermal layer to achieve genetic immunization. By varying of the particle size or acceleration speed, which is achieved by a high-voltage electric spark or a helium discharge, it is possible to influence the transfection efficiency.[73] This procedure has been mainly applied to adherent cell culture, because of the difficulty in controlling the DNA entry pathway.[85]

The most widely used strategy to deliver siRNA in vitro as well as in vivo is a pharmaceutical approach that can be subdivided into the following categories: viral and non-viral methods (Figure 4).[10, 25, 85, 96-97]

1.3.3 Pharmaceutical Methods

1.3.3.1 Viral Vectors

Most of the current gene therapy approaches make use of viral vectors (67 %), because of their nanosized flexible structure and their highly evolved technique to escape and/or bypass endocytosis with extremely high efficiency (usually >90 %).[85, 98] The viral capsid is able to associate with the endosomal membrane, which results in pore formation and lysis of the endosome.[99] Viral vectors are derived from viruses with either RNA or DNA genomes and are represented as both integrating and nonintegrating vectors.[11, 100-101] Importantly, only viruses should be considered for gene delivery that do not cause any associated pathogenic effects.[102] Generally, the enveloped virus particles, called virions, consist of their genome and a protein coat, which is sometimes surrounded of a lipid bilayer.[102] The coat can have different shapes, ranging from helical and icosahedral forms to more complex structures (Figure 6).[103-105] The first vectors, which were used in a gene therapy clinical trial on X-linked severe immunodeficiency disease (SCID-X1) and adenosine deaminase (ADA) deficiency, were retroviral vectors derived from retroviruses.[106] They are lipid-enveloped particles containing two identical copies of a linear single-stranded RNA genome of around 7–11 kb.[106] Retroviruses are found in all vertebrates, and they can be classified into oncoretroviruses, lentiviruses, and spumaviruses.[11] They are able to efficiently infect dividing cells and stably integrate their genome which is converted into a double-stranded DNA genome.[107] Since the viral entry usually requires specific membrane-bound receptors, cells which do not have the appropriate receptor are resistant to infection by a specific retrovirus.[106] The inability to infect non-dividing cells, such as lung, skin, and liver tissue, limits the use of retroviral vectors for in vivo therapeutic gene transfer.[10, 108-109]

Adenoviruses (Ad) are non-enveloped, linear, double-stranded DNA viruses that are able to

efficiently infect the mucosal linings of the respiratory tract, the eyes, the intestines, and the urinary tract[105] and express their genes in both dividing and non-dividing cells.[104] In contrast, adeno-associated viruses (AAV) consist only of a single stranded DNA genome.[110] By inserting foreign genes into Ad genomes it was demonstrated that recombinant Ad vectors could be generated and since then they have been extensively used as eukaryotic expression vectors and therapeutic gene delivery vehicles.[100]

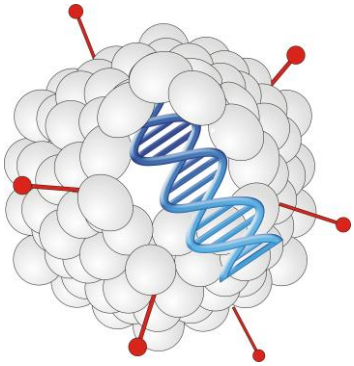


Figure 6. Structure of a virion. The virion consists of its genome, and a protein coat with glycoproteins (hemagglutinin (HA) and neuraminidase (NA)). The shape of the coat varies ranging from helical to icosahedral forms.

Virus-mediated gene delivery exhibited high efficiency since they can transduce cells in vitro with 100–10000 viral genomes per cell.[111] There are a number of concerns, however, associated with viral vector systems, including toxicity and host immune response, that limits clinical application of viral vectors which encouraged the investigation of other technologies.[100, 112]

Non-viral vectors are safer and more flexible than viral vectors, although they lack efficiency. Therefore the development of virus-like particles (VLP) has attracted a great deal of attention.[94] In this approach only the outer shell of the virus, the viral capsid, is used without any viral genetic information.[94] The first virus-like particles, which were generated by Plank et al. in 1992, contained an artificial tetra-antennary galactose ligand and a membrane-active peptide, asialoglycoprotein, that transfer DNA into hepatocytes and induce endosomal release.[113] Even though this approach resulted in efficient, ligand-specific gene expression, the result was overall inefficient in comparison to viral vector particles.[113]

1.3.3.2 Non-viral Vectors

The death of a patient in a gene therapy trial using viral vectors stopped the intermission of the clinical trial and accelerated research on non-viral vectors.[98] Compared to viral vectors, non-viral delivery systems are advantageous, because of their relative safety, simplicity of use, and the possibility of large scale quantities.[114-117] Furthermore, their DNA-carrying capacity is not limited and they can easily be modified to tailor special properties needed to efficiently apply them for cell-specific targeting.[115] However non-viral delivery systems are far less efficient than their viral counterparts, since they have to overcome cellular barriers and immune defense mechanisms.[116-119] The carrier has to pass several physiological barriers after injection and needs to travel to the specific organ or tissue in the bloodstream.

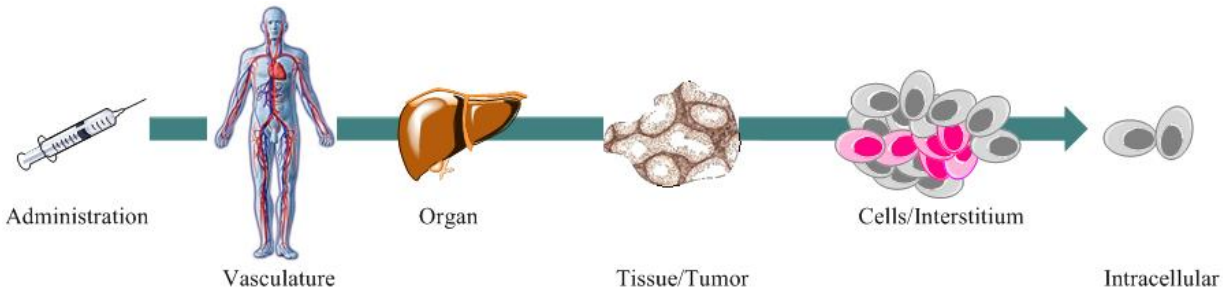


Figure 7. The systemic delivery pathway of therapeutic nucleic acid to the cells. Adapted from ref. [120]. Copyright (2005) Elsevier.

Within the tumor the carrier needs to be transported across the vascular endothelial barrier and be distributed throughout the tumor interstitium to reach the target cells where they need to be taken up efficiently (Figure 7).[41, 120]

Gene delivery systems typically encompass three length scales: (i) nano (<1µm), (ii) micro (1-10 µm), and (iii) macro (surfaces), whereas the size of each delivery system is application-dependent (Figure 8).[5]

The application of nano-sized carriers in medicine, which is referred to as nanomedicine, is an exciting new field for healthcare. With its emerging trends and recent advances exciting possibilities are openings up in biology and medicine which is potentially leading to the discovery of optimally safe and effective nanoscale delivery vehicles. The generation of nanosized particles which are being investigated to enhance the delivery of nucleic acids are called generally "nanocarriers". As the word "nano" means one billionth of a meter, nanoscience tries to understand and manipulate the properties of matter and living systems on nanometer length scale (Figure 8).[121]

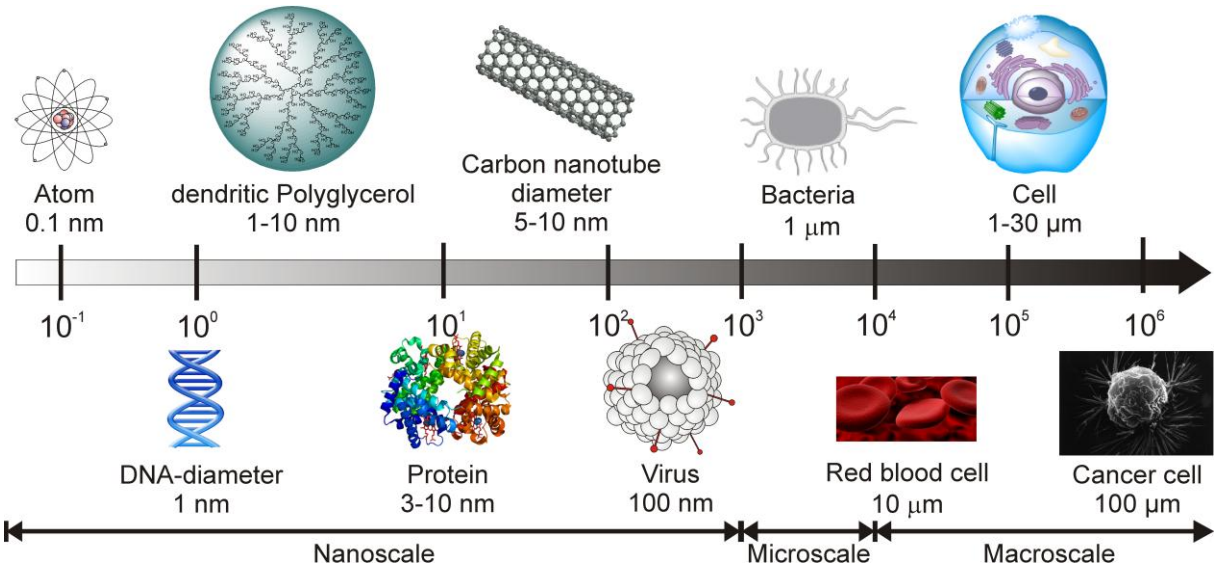


Figure 8. The size of nanotechnology in biology and chemistry. Adapted from ref. [122].

Even after the cellular uptake, the delivery vehicles have not completed their task because the nucleic acid still has to be carried along the intracellular medium involving endosomes and lysosomes.[123] Therefore the nanoscale delivery vehicles have to help the nucleic acid to escape from the endosome before reaching the lysosomes that affect the degradation of the nucleic acids.[124] Finally the therapeutic gene must to be released from the carrier itself to reach the desired target cell compartment.[39, 41, 62, 125]

A large number of non-viral systems, most of the currently available chemical methods rely on two main components, cationic lipids and cationic polymers.[97, 114, 125-129] Both must surmount the barriers to gene delivery and both have been shown to offer potential routes to efficiently deliver nucleic acids into the cell.[116, 130] These cationic compounds spontaneously interact with DNA by electrostatic interactions between positively charged amine groups of polycations and negatively charged nucleic acids to form particles of virus-like dimensions (less than 250 nm) which is a prerequisite for efficient transport through the cell membrane.[42, 66-67, 114, 116, 118, 131-133] Since these complexes carry extra positive charge on their surfaces they facilitate binding to cellular anionic proteins (glycoproteins, proteoglycans, and glycerolphosphates) and mediate the cellular entry by non-specific endocytosis as illustrated in Figure 9.[29, 61, 127, 134] Furthermore, these particles, known as lipoplexes or polyplexes can protect the condensed genetic material to overcome biological obstacles, including undesired interactions with blood components associated with systemic delivery.[116, 118, 135-139]

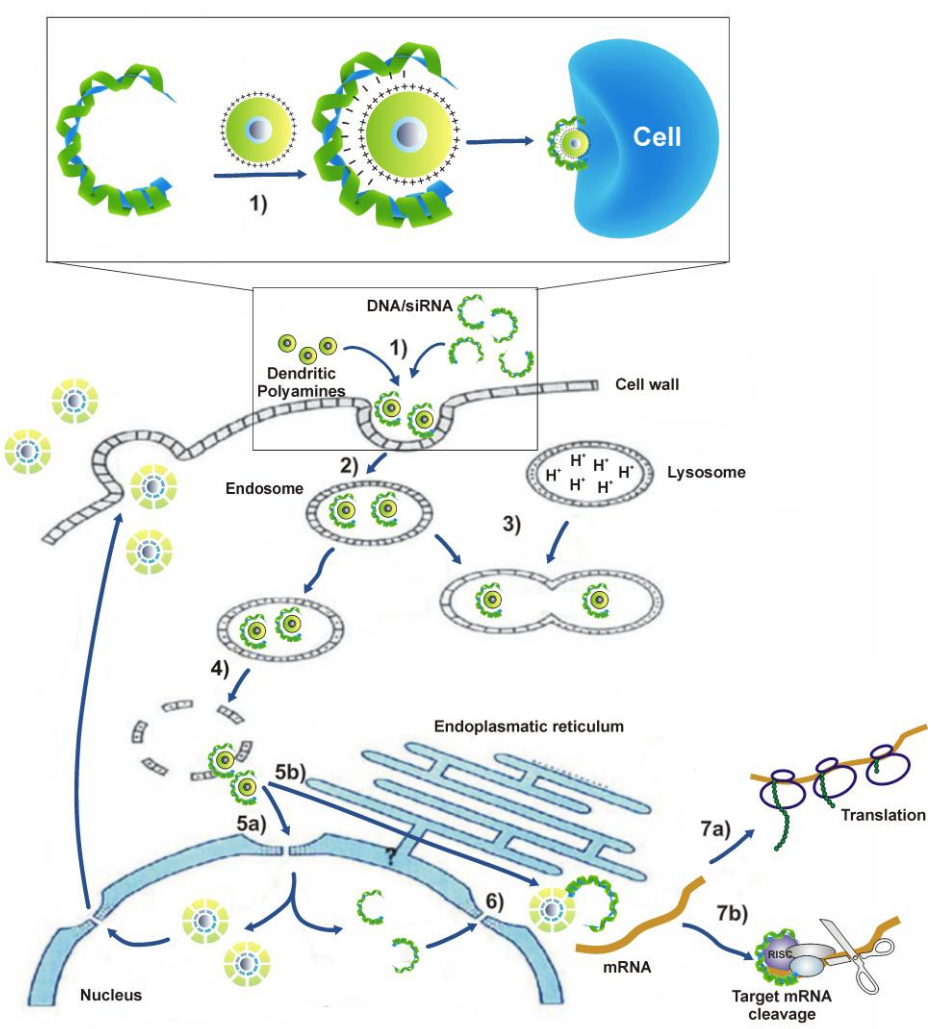


Figure 9. Proposed mechanism of DNA/siRNA delivery in the form of dendriplex and principle of polyplex formation. (1) Formation of the DNA/polymer complex (polyplex), (2) endocytosis of the polyplex, (3) fusion of endosome and lysosome, (4) endosomal release of the polyplex into the cytosol, (5a) incorporation of the polyplex into the nucleus, (5b) release of the siRNA into the cytosol, (6) transcription of the DNA into mRNA followed by release of the polyamine back into the cytosol, (7a) translation of mRNA, and (7b) mRNA degradation. The metabolism of the polyamine is still unclear. Adapted from ref. [140]. Copyright (2002) Elsevier.

Since single-protonated gene vehicles typically interact through monovalent binding with the DNA, the interaction is relatively weak and competes with salt binding under biological conditions. Therefore polymers with multiple positively charged groups are under investigation. Polymers typically improve the potential of the respective nucleic acid, since they circumvent limitations, such as short half-life in the blood stream and a high overall clearance rate. Furthermore, the reason for using polymers relies on the enhanced permeability and retention (EPR) effect,[64] a phenomenon associated with tumor vasculature and the lymphatic system.[141] Since tumor tissues grow faster than normal vessels, tumor vasculature is distinctly abnormal in structure and function and is characterized by larger

exaggerated gaps, tortuosity, and hyperpermeability.[142] In general, only small molecules are able to accumulate in healthy cells, whereas larger molecules cannot penetrate the dense endothelia cell layer at all, which results in passive accumulation.[141] Since defective tumor endothelial cells lead to abnormal molecular and fluid transport dynamics, there is a selective extravasation and tumor accumulation of proteins or nanoparticles.[64]

1.3.3.2.1 Cationic Lipids

Cationic lipids are amphiphiles that consist of three structural domains: the positively charged hydrophilic head group, two lipophilic tails or a steroid moiety, and a connecting linker between the two domains.[130, 143-145]

Cationic lipid-based transfection reagents were first introduced by Felgner et al. in 1987.[146] Since they are non immunogenic and can be easily prepared in large quantities, lipids are used as gene delivery agents.[94, 137] The first lipid used in gene therapy was DOTMA (N-[1-(2,3-dioleoyloxy)propyl)]-N,N,N-trimethylammonium chloride) which consists of a quarternary amine head group which is connected via ether groups to two unsaturated aliphatic alkyl chains.[130, 137, 147-149] Another cationic lipid commonly used in gene therapy is the ester-linked variant DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane) shown in Figure 10.[130, 150]

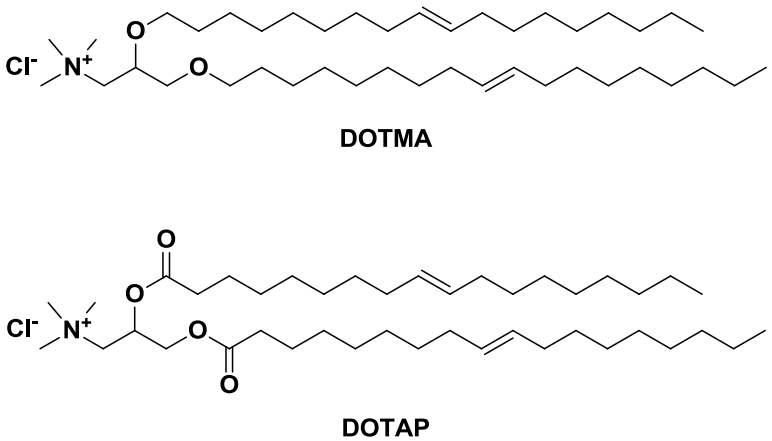


Figure 10. Chemical structures of commonly used cationic lipids DOTMA and DOTAP.

These amphiphiles can spontaneously self-assemble to form non-covalent lipoplexes through electrostatic interactions between the positively charged polar head group and a negatively charged phosphate group of the nucleic acid (Figure 11).[151-152] The lipoplexes can shield the DNA from enzymatic degradation and the overall positive charge facilitates binding

to cellular membrane.[29, 39, 151, 153] After the uptake, lipoplexes trigger endosomatic release by provoking membrane perturbation with the result that the DNA can be intracellular released by incorporated pH-, redox-, or enzyme-sensitive linker moieties.[130, 151, 154]

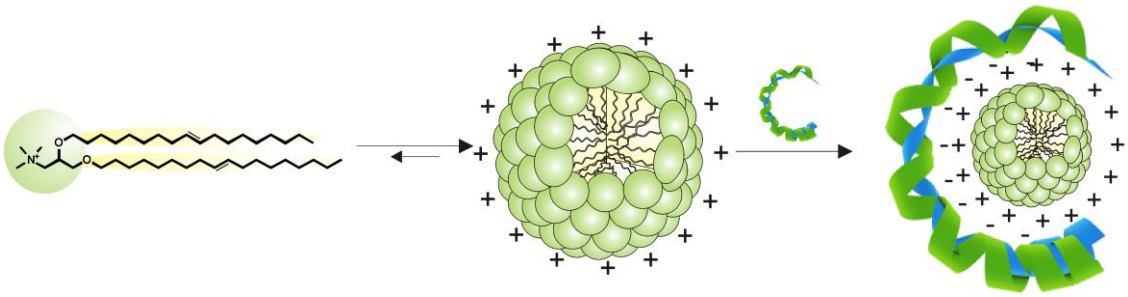


Figure 11. Structure of the cationic lipid DOTMA, aggregation of lipid amphiphiles, and proposed DNA condensation.

Since it is known that the gene transfection efficiency of cationic lipids relies upon physicochemical characteristics, and since all constituent parts of the lipid can be easily modified, they have become more and more attractive and a range of these positively-charged amphiphiles have been reported for transfection of plasmid DNA such as DOGS (dioctadecylamido-glycylspermine), DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxamido)-ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate) known as well as Lipofectamine®, DOTAP and DOTMA.[130] Another lipid-based transfection reagent, called HiPerFect®, was especially synthesized for transfection of siRNA. All these reagents are only used for in vitro experimentations and they are mainly used to transfer genetic material to solid cancer cells or respiratory epithelial cells.[147]

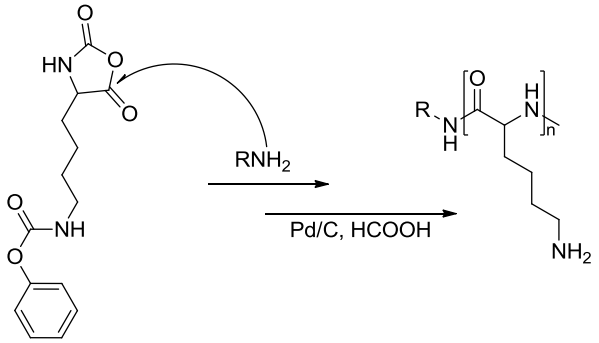
The major disadvantages in the in vivo use of cationic lipids are their low transfection efficiency that is attributed to their heterogeneity and its instability in physiological (i.e. serum containing) environments.[114] However, cationic lipids are the most frequently used transfection agents for DNA and siRNA delivery in vitro.[151]

1.3.3.2.2 Cationic Polymers

Polymers can be specifically designed with different length, molecular weight, and scaffolds ranging from linear to highly dendritic architectures.[26, 155-163] Furthermore, they can be easily modified with substitutions or additions of functional groups or by cell- or tissue-specific targeting moieties and are completely soluble in water.[164] For efficient complexation of

nucleic acids via electrostatic interaction, cationic polymers are used that acquire their charge from primary, secondary, tertiary, and/or quaternary amino groups.[152] In contrast to cationic lipids, cationic polymers are able to more efficiently condense nucleic acids, which causes localized bending that results in the collapse of the genes into rods, spheres, or toroids.[165] Since these complexes carry a positive charge on their surfaces, they easily absorb the negatively charged cell surface and mediate the cellular entry by non-specific endocytosis.[29, 61, 127, 134] Furthermore, these polycations, so-called "proton sponge" polymers, are able to induce membrane destabilization at acidic pH which is based on the fact that polyamines contain a large number of secondary amines and exhibit pKa values between physiological pH (7) and lysosomal pH (5-6).[166] During endocytic trafficking, these polymers get protonated and the accumulation of protons in the vesicle must be balanced by an influx of counter ions followed by osmotic swelling and finally rupture of the endosome which releases the polyplexes into the cytosol.[118] The general mechanism of polyamine mediated gene transfection is illustrated in Fig. 9.

Pioneering gene therapy with cationic polymers has been conducted with PLL (poly($_L$-Lysine) since the formation of polyelectrolyte complexes between PLL and RNA was identified.[1, 167] Due to its natural amino acid backbone, PLL is a biodegradable polymer, which makes it especially suitable for in vivo applications.[137] Because the primary amino groups of PLL are positively charged at physiological pH, PLL can interact electrostatically with the negatively charged nucleic acids to complex them into polyplexes of around 100 nm.[168-169]



Scheme 2. Synthesis of PLL via ROP of protected N-Carboxy-(N-benzyloxycarbonyl)-$_L$-lysine anhydride. Adapted with permission from ref. [16]. Copyright (2009) American Chemical Society.

PLL is synthesized by ring-opening polymerization (ROP) of protected N-Carboxy-(N-benzyloxycarbonyl)-$_L$-lysine anhydride as shown in Scheme 2, whereas the ratio between initiator and monomer determine the average degree of polymerization and average molecular weight. One of the earliest reports on ring-opening polymerization (ROP) was presented in

1992 by Suzuki et al.[170] who described palladium-catalyzed polymerization of a cyclic carbamate. The polymerization was proposed to be an in situ multibranching process, wherein number of propagating chain ends increased with the progress of polymerization.

To evaluate the gene transfection efficacy, many PLL polymers with different molecular weights were tested.[137] In general, PLL structures with a molecular weight of less than 3000 could not form stable complexes with DNA, indicating the importance of primary amine number for complex formation.[171] Although it has been shown that the DNA complexation capability and the transfection efficiency of PLL increased with high molecular weight, PLL/DNA polyplexes showed undesirable high toxicity.[137] PLL/DNA polyplexes have a highly positive ζ-potential and are therefore able to rapidly bind to the negative charged cell surface and they are efficiently taken up by absorptive endocytosis.[169]

Since PLL has a lack of amino groups with a pKa between 5 and 7, it has no buffering capacity to allow the endosomal escape. As a result, the gene expression using PLL alone or without modifications was rather low.[29, 172] To enhance the transfection efficiency the inclusion of co-application of endosomolytic agents like chloroquine, a lysosomotropic agent is required.[29, 137, 173]

Polyamidoamine (PAMAM, „Starburst“) dendrimers are the first type of polycationic molecules with high transfection potential in the absence of endosomolytic agents and are the most common class of dendritic macromolecules (Figure 13).[169, 174] PAMAM dendrimers consist of a multifunctional alkyl-diamine residue, tertiary amine branches that exert endosome buffering effects, and primary amino groups which participate in DNA binding due to their pKa values.[175-176]

After Staudinger initiated the synthetic macromolecular revolution approximately eighty years ago with the introduction of the ‘macromolecular hypothesis’, there were only three major polymer architectures/classes, namely, linear, cross-linked, and branched types.[177]

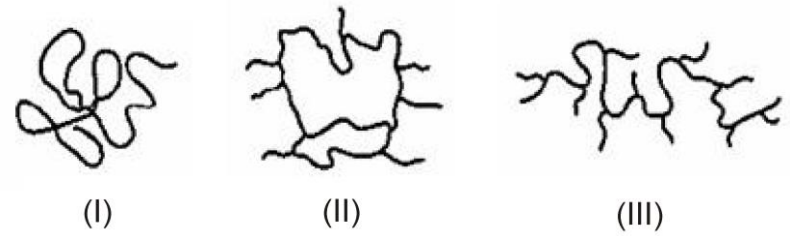


Figure 12. Three major classes of macromolecular architectures organized chronically according to their development: (I) linear, (II) cross-linked (bridged), and (III) branched. Adapted from ref.[177]. Copyright (2011) Elsevier.

In general, all these architectures were largely produced by statistical polymerization processes. Therefore their architecture was not structure-controlled like those observed in biological systems.[177] The major macromolecular architectures are presented in Figure 12.

However, since the pioneering work in constructing the three-dimensional, perfectly branched macromolecules of Vögtle, Tomalia, and Newkome, a new class of macromolecular architecture, the dendritic architectures (VI), has changed this paradigm. Dendrimers are nearly perfect monodisperse, three-dimensional macromolecules with a well-defined tree-like globular structure and a large number of functional groups.[178-180] They consist of three major architectural components: core, branches, and end groups.[179] Their size, shape, and reactivity are determined by generations [=G]/ shells and architectural components. They are produced in an iterative sequence of reaction steps, in which each additional step leads to a higher generation material.[181] Two synthetic approaches for the synthesis of dendritic architectures are possible which are described as *divergent* and *convergent*.[182] In the divergent synthesis, dendritic construction results from a core and proceeds outward toward the macromolecular surface, whereas in the convergent approach, branched polymeric arms are grown from "outside in".[178-185]

In the beginning, the term "dendrimer" which was established by Tomalia in 1985[181, 186] described all types of dendritic polymers but later a distinction based on the relative degree of structural control present in the architecture was drawn.

In transfection studies with PAMAM Szoka et al. found that his previously reported high transfection levels could not be reproduced. Furthermore he could show that stringently synthesized and purified PAMAM was 100-fold less active than partially degraded (= hyperbranched) PAMAM.[134, 187]

A comparison of the resulting DNA complexes formed by each polymer, whether perfect or hyperbranched, showed that both forms compact DNA structures of nearly the same size, which lead to the conclusion that the key for optimal transfection activity correlates to the polymer flexibility.[120]

Hyperbranched polymers are highly branched molecules composed of dendritic (D), linear (L), and terminal units (T) that represent a compromise between the perfect structures of dendrimers and the partially degraded architectures.[24]

Instead of the tedious step-wise synthesis of perfect dendrimers, hyperbranched polymers are prepared in a one-step synthetic strategy.[188] Due to their similar physicochemical

properties dendrimers and hyperbranched polymers are referred to as dendritic polymers in the literature.[2]

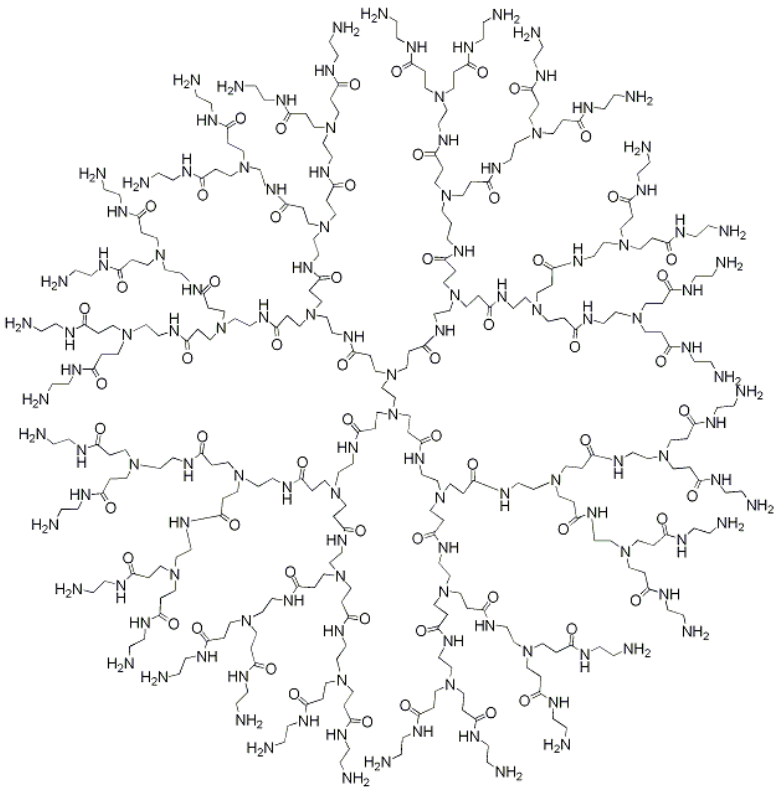


Figure 13. Chemical structure of [G4]-PAMAM dendrimers.

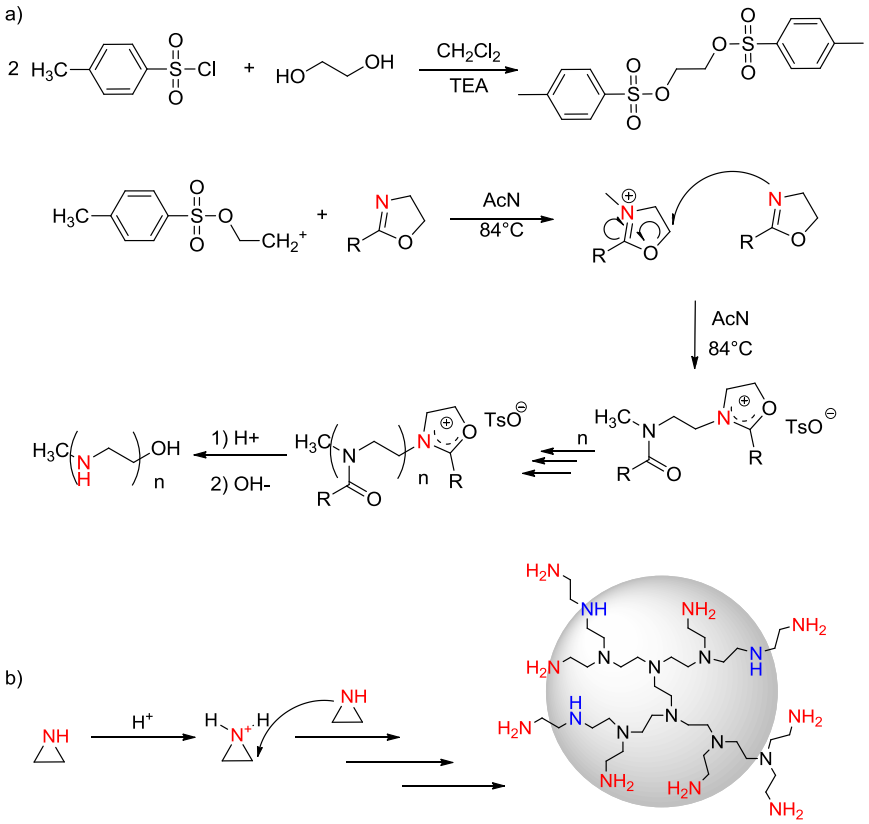
Besides generation number and degree of flexibility, hyperbranched PAMAM can be synthesized with different chemical properties. Two hyperbranched PAMAM dendrimers, known as Superfect® and Polyfect® are commercially available from QIAGEN (Hilden, Germany) as in vitro transfections agents.[94] Although hyperbranched PAMAM are tolerated in many cell lines in vitro, their in vivo applications are still limited due to significant toxicity.[189]

Since the first successful transfection studies, poly(ethylene imine)s (PEI)s have become the most commonly used polymers for gene transfection. PEIs are some of the most powerful and versatile scaffolds and range from linear,[190-191] branched,[192] and hyperbranched[193-194] to dendrimers.[193] PEIs are capable of efficiently transfecting nucleic acids in vitro as well as in vivo and have even been considered as the gold standard of non-viral delivery systems.[16, 156, 160] Hyperbranched PEI is commercially available and has already been produced for almost 50 years on a ton scale by BASF (Lupasol®),[195-196] but the first PEI-mediated gene transfer was conducted by Behr et al. in 1995.[197] The main advantage of using PEIs is that they offer a significantly higher efficiency in transfection and protection against

enzymatic degradation than other polycations, such as poly(L-lysine), which is possibly due to their higher charge density and more efficient complexation.[29] Such complexation of DNA is facilitated by electrostatic interactions between the positively charged amino groups of PEI and the negatively charged phosphate groups of DNA.[62] The condensed DNA particles have a highly cationic surface charge which is required for efficient binding to the cell surface and uptake through endocytosis.[94]

Furthermore, the protonable amino groups make the polymeric scaffold an effective "proton sponge" at virtually any pH[156] which allows the endosomal escape. Therefore gene expression using PEI is likely one of the crucial factors for the high transfection efficiencies obtained with these polymers.

Linear PEI can be obtained by ring-opening polymerization of oxazoline and its derivatives (Scheme 3a),[155] whereas hyperbranched PEI is typically synthesized via an acid catalyzed, ring-opening polymerization process of aziridine (ethylene imine) at 90–100 °C in water or organic solvents (Scheme 3b).



Scheme 3. Synthesis and structure of PEI (a) synthesis of linear PEI[161] and (b) synthesis of hyperbranched PEI[2] via acid catalyzed, ring-opening polymerization of aziridine. Each color represents a different branching unit: blue for linear units (L), black for dendritic units (D), red for terminal units (T). PEI has a degree of branching (DB) of 62–73 %, the depicted structure represents only a small idealized fragment. 3a) Adapted from ref. [161]. Copyright (2010) John Wiley and Sons.

Hyperbranched PEIs can be obtained with narrow molecular weight distributions (MWD, typically below 2.0)[195] and molecular weights (Mn) up to 10,000 g/mol, which corresponds to the typically used MW PEI (25 kDa, with PD of about 2.5). Higher molecular weight PEIs are accessible via crosslinking with bifunctional compounds such as 1,2-dichloroethane.

A variety of PEI derivatives have already been tested and numerous physical characterizations of PEI/DNA complexes including size, shape, surface charge, and concentrations for their gene transfer efficiency have been performed. Additionally, PEI has been successful for local in vivo applications in gene delivery to a variety of tissues, including the central nervous system,[190, 198] kidney,[199] lung,[200-201] and tumors.[202] Local application represents a promising approach, since several barriers of systemic application can be avoided.[29]

Interestingly, PEI of different molecular weights and degree of branching differ with respect to their complexation behavior, in vivo transfection efficiency, and toxicity.[97] Highly branched PEIs that form smaller polyplexes usually achieve higher transfection efficiencies, yet also have greater toxicity, since PEIs are non biodegradable and the cytotoxicity tends to increase with high molecular weights and with increased branching.[139, 203] Therefore, the major drawback of PEI is its toxicity, both in vitro and in vivo, due to the excessively high charge density.

1.3.4 Chemical Modification

Unmodified and naked administered siRNA are highly vulnerable to serum exo- and endonucleases, leading to a half-life time of less than an hour in human plasma serum. Furthermore, unmodified siRNA do not easily cross cell membranes and they have limited tissue distribution. As a result, a wide variety of chemical modifications have been proposed to improve the serum stability, their intracellular penetration, and bioavailability.[45, 63, 204-210] Since most siRNAs were produced through chemical synthesis, it is technically possible to incorporate various modifications into siRNA backbones.[211] In principle, three sites for chemical modifications can be distinguished (Fig. 14): analogs with unnatural bases, modified sugars (especially at the 2'-position of the ribose, (Figure 15 a-e) or altered phosphate backbones (Figure 15 f-i).

The use of unnatural nucleotides presents a particular challenge, because the modifications must not interfere with the silencing activity of the siRNA. Several studies have shown that the silencing activity depends on the position of the modification in the sequence of the

antisense strand.[212] Remarkably, a free 5' hydroxyl group of the antisense strand is required for entry of the siRNA into the RNAi pathway.[210]

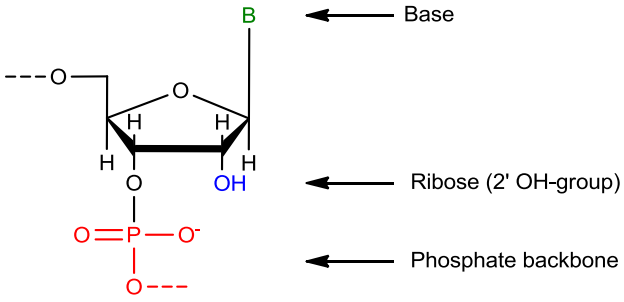


Figure 14. Sites for chemical modifications of ribonucleotides. Adapted from ref. [210]. Copyright (2003) John Wiley and Sons.

The most useful modifications are on the ribose moiety. Since the 2'-position of the ribose do not affect the RNAi machinery, these position have been extensively modified to improve the stability of the siRNA by linkage of 2′-fluoro (2'-F), 2′-O-fluoro-β-D-arabinonucleotide (FANA), locked nucleic acid (LNA), which contains a methylene bridge connecting the 2′-O with the 4′-C of the ribose ring, or 2′-O-methyl (2'-OMe).[211]

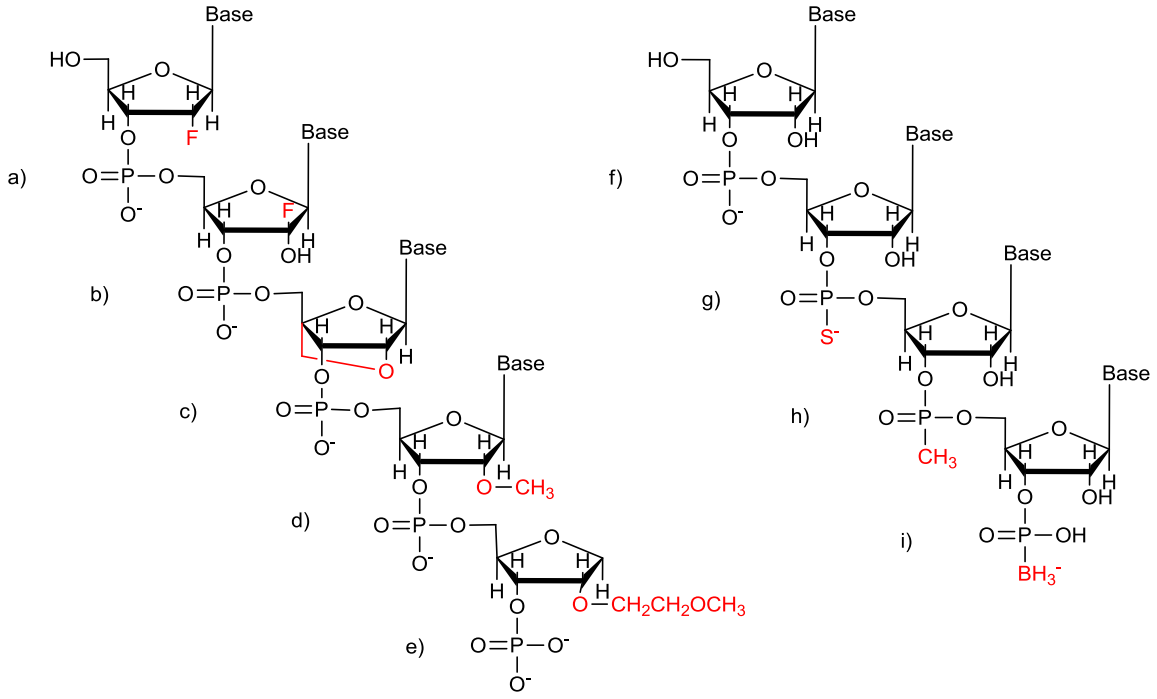


Figure 15. Chemical modifications that can be incorporated into siRNAs. Selected modifications are shown to the sugar backbone (a-e), including (a) 2'-flournucleotid, (b) 2-fluoro-β-D-arabinonucleotide (FANA), (c) locked nucleic acid (LNA), (d) 2'-O-methyl-RNA (2'OMe) and (e) 2-O-(2-methoxyethyl)-RNA (2'O-MOE). Modifications to the backbone are shown (f-i), including (f) native RNA backbone, (g) phosphorothioate, (h) methylphosphonate, and (i) boranophosphate. Adapted from ref. [211]. Copyright (2011) Elsevier.

Several variations on the non-bridging oxygen in the phosphodiester linkage are also accepted by the RNAi machinery. The phosphodiester could be replaced, for example with sulfur (phosphorothioate), methyl (methylphosphonate) groups, or boron (boranophosphate). Even if these modifications were fundamentally tolerated by the RNAi machinery, and the sense and antisense strands showed significantly enhanced resistance towards degradation by exo- and endonucleases in serum, toxic side effects could be observed. For example, siRNA with 50 % phosphorothioate content displayed cytotoxic properties and reduced cell growth and viability.[213] The modifications can often lengthen the half-life of the siRNA in plasma, ranging from 1.5 h [54] up to 6.5 h.[207]

Another possibility would be to structurally modify in the heterocycles to strengthen base-pairing and thus stabilizing the duplex formation between antisense siRNA and their target mRNA.[210]

For successful delivery of polymer conjugated/complexed genetic material, several cellular phenomena like endocytosis and endosomal escape act as governing factors. These phenomena and their implication in gene therapy will be explained in the following section.

1.4 Endocytosis

The cell membrane is a fluid mosaic of lipids, proteins, and carbohydrates that segregate the cytoplasm from the extracellular environment.[214] The membrane consists of three different major classes of lipids, namely, phospholipids, cholesterol, and glycolipids.[214] The amount of each lipid depends upon the type of cell.

Lipids are structurally asymmetric due to their hydrophilic head and their hydrophobic tails. The polar head regions are used to anchor proteins across the bilayer, ionic amino acids or saccharids, and are in contact with the aqueous phase. The membrane is selectively permeable and regulates the entry and the exit of small and large molecules.[214] Because the interior of the lipid bilayer is hydrophobic, the polar molecules cannot enter the cell (Figure 16).[214]

However, cells devised means of transferring small molecules, such as amino acids, sugars, ions, and polar molecules, which are able to traverse the cellular membranes through integral membrane protein pumps or channels, whereas the protein-free lipid bilayers are highly impermeable to ions.[215] Most macromolecules, such as nucleic acids or polyplexes are not taken up by the cell spontaneously.[94, 214]

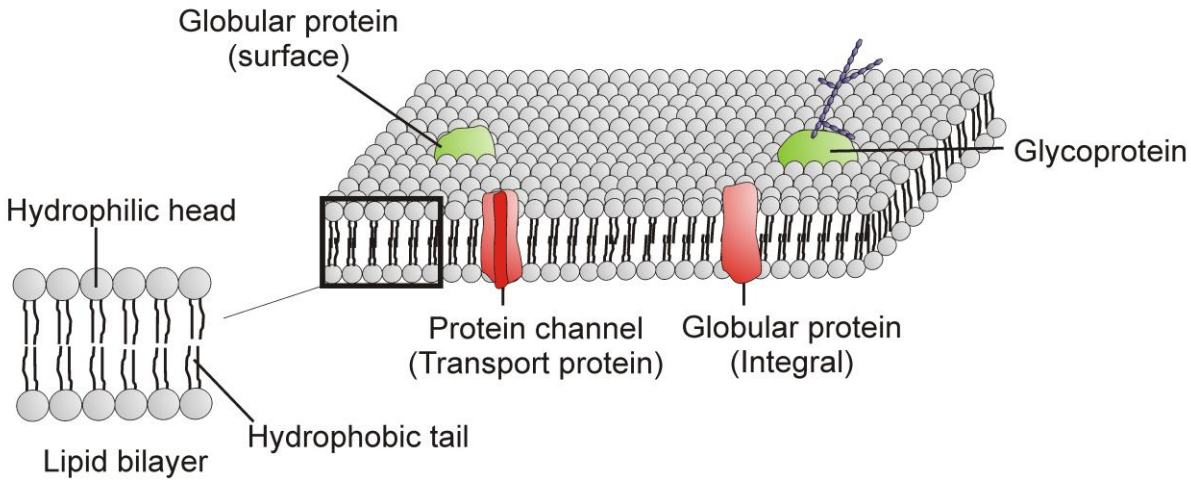


Figure 16. The fluid mosaic model of the structure of cell membranes.

Due to the positive surface charge polyplexes are able to bind efficiently to the cell through electrostatic interactions with negatively charged membrane components.[61] Although the exact mechanism of the uptake of the cationic polyplexes is still not completely elucidated, current evidence suggests that the polyplexes are taken up by means of endocytosis.[138] In general, the material to be internalized is surrounded by an area of plasma membrane, which then buds off inside the cell to form a vesicle. Mammalian cells have evolved a variety of different mechanisms to internalize particles from the surrounding medium.[216] Collectively these mechanisms are termed endocytosis. Endocytic mechanisms control many important cellular functions including the nutrient uptake, migration, receptor signalling, receptor downregulation, neurotransmission, and pathogen entry.[217] Endocytosis may occur in various ways, including adsorptive or fluid phase endocytosis (cell drinking), receptor mediated endocytosis, macropinocytosis, or phagocytosis (cell eating).[114]

Phagocytosis usually occurs only in specialized cells, such as macrophages and involves the uptake of larger particles (Figure 17).[218] There are four mechanisms for pinocytosis which occurs in all cells. They are macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, and clathrin- and caveolae independent endocytosis (Figure 17).[219] In each case the cargo molecule and its receptor determine the pinocytic pathway through which they gain entry into the cell.[219]

Macropinocytosis usually occurs from highly ruffled regions of the plasma membrane that is induced in many cell types upon stimulation by growth factors or other signals.[114] These stimuli trigger the invagination of the cell membrane around the cargo, that collapse onto and fuse with the plasma membrane to generate large endocytic vesicles called macropinosomes.[216, 219] These vesicles contain large amounts of fluid and membrane.

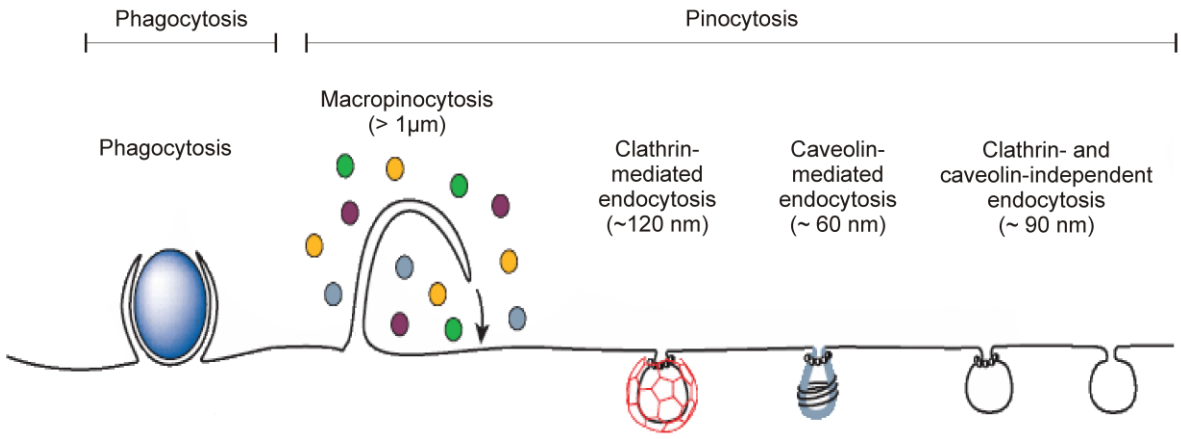


Figure 17. Schematic representation of several endocytoic pathway mechanisms. The endocytic pathways differ with regard to the size of the endocytic vesicle, the nature of the cargo (ligands, receptors and lipids), and the mechanism of vesicle formation Adapted from ref. [219]. Copyright (2003) Nature Publishing Group.

Clathrin-mediated endocytosis (CME) is crucial for intercellular communication during tissue and organ development and is the most important pathway in cells of higher eukaryotes for receptor dependent uptake of growth factors and nutrients.[219] Clathrin is a cytosolic coat protein with a three-legged structure that is able to build up a clathrin forming a net-like basket.[220-221] CME occurs in specialized regions of the plasma membrane, called clathrin-coated pits that involve the concentration of high-affinity transmembrane receptors.[219] Coated pits can be internalized to form clathrin-coated vesicles (CCV) that are approx. 60-80 nm in diameter and can concentrate large extracellular molecules.[222] These molecules bear different receptors that are responsible for the receptor-mediated endocytosis of ligands, e.g., low density lipoprotein (LDL), transferrin, and insulin.[223]

Internalization can also take place by another class of vesicles that are coated with the protein caveolin, a cholesterol binding protein.[219] The corresponding mechanism is called caveolae-mediated endocytosis.[224] Caveolae (caves), appear as flask-shaped, cholesterol and sphingolipid rich smooth invaginations, that are derived from lipid rafts, rigid membrane microdomains and are the most commonly reported non-clathrin coated plasma membrane buds.[225] Caveolae are subdomains of glycolipid rafts and are internalized via a common, clathrin independent, dynamin dependent, and cholesterol sensitive pathway.[226] They are around 50-80 nm in diameter and can constitute approximately a third of the plasma membrane area of the cells of some tissues, and are especially abundant on the surface of endothelial and in smooth muscle cells. Interestingly, multicaveolar assemblies can be observed, where many caveolae are connected together.[222]

Besides these pathways, there is evidence that cells possess clathrin and caveolae independent endocytosis pathways.[217] These endocytic pathways require specific lipid compositions, so-called rafts, that provide a physical basis for specific sorting of membrane proteins and/or glycolipids based on their transmembrane regions.[219] These small rafts diffuse freely on the cell surface and can be internalized within any endocytic vesicle.[219] Entry of the polyplexes into the cell seems to occur mostly by unspecific as well as clathrin- and caveolae-dependent endocytosis, but the mechanism is still not completely elucidated, since the uptake pathway seems to vary for different cell and polyplex types.[138] Van der Aal et al. reported that uptake of pDMAEMA and PEI polyplexes in COS-7 cells is mediated via different uptake routes, including both the clathrin- and caveolae-mediated pathway,[226] whereas Gabrielson et al. have shown that successful PEI-mediated gene delivery proceeds via an unspecific process.[227] However, size, charge density, flexibility, and amphiphilicity, as well as the structure can have an influence on the endocytoic pathway.[219]

In addition to nontargeting vector/DNA polyplexes, different vector systems were developed to promote delivery into specific cell populations by incorporation of cell targeting moieties into the vector system.[168, 228-231] These moieties/ligands (e.g., transferrin,[168, 232-239] epidermal growth factor (EGF),[236, 240] arginine-glycine-asparagine (RGD),[241-245] and folate),[242, 246-250] recognize cell type specific receptors in order to direct cellular uptake via receptor-mediated endocytosis, as illustrated in Figure 18. Once bound to the receptors on the cell surface, the vector/DNA polyplexes are internalized by clathrin-dependent endocytosis. Since the cellular uptake is mediated by receptors, the process is known as receptor-mediated endocytosis.[251]

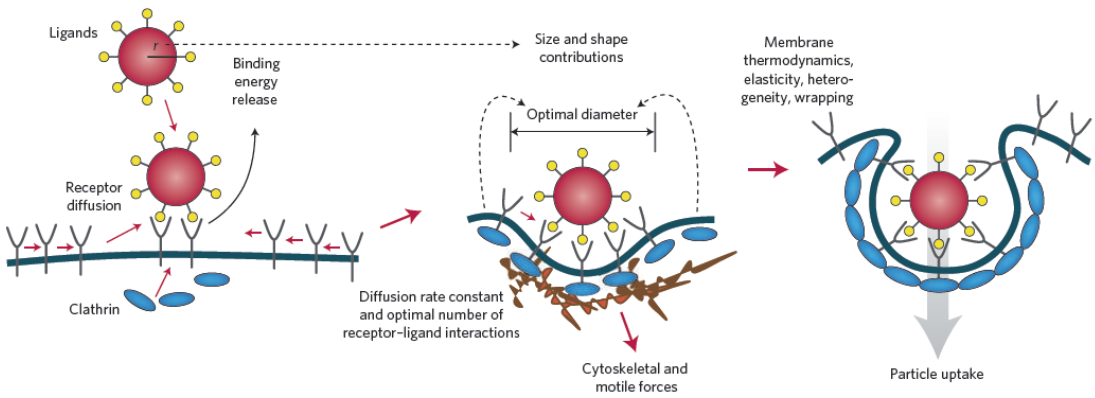


Figure 18. Receptor-mediated endocytosis. Nanocarriers (red spheres) with attached ligands (yellow dots) bind to Y-shaped receptors and wrapping at the surface membrane. Adapted from ref. [252]. Copyright (2010) Nature Publishing Group.

The cellular uptake and expression of DNA complexed with transferrin (Tf)-poly($_L$-lysine) conjugates is called *transferrin*fection. Transferrin is an iron-binding glycoprotein (79kDa) that has been used as a tumor-targeting ligand since Tf receptors are overexpressed on the surface of rapidly dividing tumor cells due to the increased cellular need for iron.[168] Moreover, Tf which is conjugated to a vector/DNA polyplex, exerts an additional shielding function that based on its relatively large size and on its slightly negative charge which prevents unspecific interactions with blood components.[235]

The epidermal growth factor receptor (EGFR) is a small protein (6 kDa) that is a rational target for antitumor strategies, since it is strongly overexpressed in a variety of human tumors of epithelial origin. The EGFR signalling pathway is one of the most important pathways that regulate growth, survival, proliferation, and differentiation in mammalian cells.[253-255]

Several peptides with the arginine-glycine-asparagine (RGD) sequence have been developed to target integrin receptors that are expressed on the activated endothelial cells in tumor vasculature. These peptides have a cyclic RGD domain that is able to efficiently recognize the integrin receptor in a variety of cell types.[256-257]

The folate receptor is another example of receptors overexpressed in tumor cells, and can therefore be used as well for tumor targeting. Transferrin and folate are among the most popular targeting ligands used for promoting tumor cell uptake.[114, 258-259]

1.5 Endosomal Escape

The efficient delivery of therapeutic macromolecules to the cytoplasm is often the weak link of gene therapy and is limited by their intracellular trafficking pathway.[166] After endocytotic uptake the nucleic acids have to travel through the intracellular medium involving endosomes and lysosomes.[133] In order to reach their target, the endosomal membrane could be a major biological barrier that should be overcome in order to deliver the nucleic acid to the cytosol before reaching the lysosomes that affect their degradation, because they contain various degradative enzymes including proteases, nucleases, and lipases (Figure 19b).[260] Several strategies have been tested to facilitate endosomal escape, either by endosomal escape agents or structural diversities.[39, 156, 261-263]

1.5.1 Endosomal Escape Agents and Mechanisms

Several approaches have been tested to especially facilitate early endosomal escape, like the pH-buffering effect (proton sponge effect). The ability to escape the endosome generally requires membrane-destabilizing agents, which can be subdivided in viral and bacterial proteins and chemicals. The last category includes such chemicals like chloroquine and ammonium chloride that buffer acidic vesicles thus inhibiting the enzymes involved in lysosomal degradation (Figure 19a).[264-265] Chloroquine is a low molecular weight lysosomotropic agent that has to be incorporated into the DNA/vector complex to enhance non-viral transfection efficiency.[16, 156, 158, 173-174, 263-264] Chloroquine and ammonium chloride are relatively lipophilic in their unprotonated form and can therefore easily penetrate membranes. After entering an acidic environment they become protonated and finally too polar to escape. If they do enter the pH gradient lipoplex/polyplex and trigger the drug release from within or if the pH increase triggered by chloroquine contributes to gene release is still not clear.[248]

Many non-viral vectors like polyethylenimines or polyamidoamines as well as several cationic lipids provide a high transfection efficiency per se. They have amine groups with low pKa values that can utilize the acidic environment of endosomes (pH=5.0–6.5).[166] Those vectors which are endosome-disruptive under slightly acidic conditions and which are nondisruptive at physiological conditions (pH=7.4) are thought to act by a proton-sponge effect.[166] Polyamines which are the most investigated scaffold demonstrate the effect of endosomal escape on gene delivery. One example is PEI, which is able to mediate relatively high levels of gene transfer on its own.[156] PEIs, are one of the most powerful and versatile classes of scaffolds, range from linear,[190-191] branched[192] and hyperbranched[193-194] to dendrimers.[193] This allows the compaction of DNA into small particles which facilitates the endocytosis as well as preventing the DNA from endosomal disruption in the absence of any exogenous endosomolytic agent.[116] Branched PEIs show a high charge density and possess primary, secondary, and tertiary amino groups in a 1: 2: 1 ratio.[264] These amines exhibit pKa values between physiological and lysosomal pH.[266] Since PEI is only partly protonated at physiological pH, the availability of protonatable nitrogens results in buffering capacity.[267]

In fact, nearly every third atom of these polymers is an amino nitrogen which can be protonated. Therefore these polymers undergo large changes in protonation during endocytic trafficking.[268] The degree of protonation of the amines increases from roughly 20 to 45 % as pH decreases from pH 7.2 and 5.0.[264] PEI-based transfection agents show high transfection

efficiency, which is proposed to be due to the above-mentioned "proton sponge effect".[29, 98] After endocytosis the endosome is acidified by the lysosomal proton pump, the ATPase enzyme that actively transports protons from the cytosol into the vesicle to reach pH 5–6.[269] The accumulation of protons in the vesicle must be balanced by an influx of counter ions to maintain charge neutrality.[118] Moreover, internal charge repulsion due to PEI protonation and the increased ionic strength inside the endosome cause swelling and ultimately rupture, leading to polyplex deposition into the cytosol.[12] The "proton sponge" enhances DNA transfection, which causes a faster release of the DNA and with the result that degradation of the DNA strands can be avoided.[197, 264] The proton sponge effect is illustrated in Figure 19a.

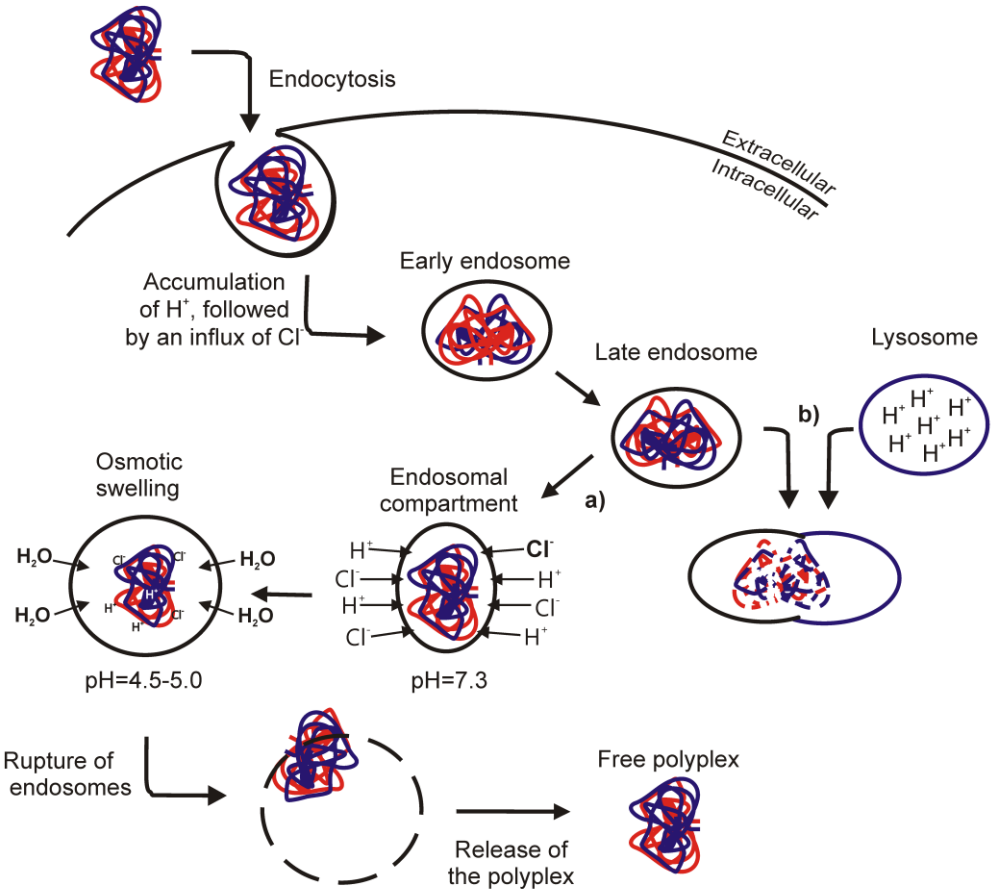


Figure 19. Internalization of nucleic acids into the cell through endocytosis and subsequent endosomal escape. Early endosomes consist of the vesicles containing the nucleic acids coming from the cell surface. Late endosomes, which are thought to mediate a final set of sorting events prior to interaction with lysosomes, receive the internalized materials from early endosomes. The endosomal release (a) requires a membrane-destabilizing agent or amine groups with low pKa values that causes a proton sponge effect. The proton sponge hypothesis: H^+ and Cl^- entry into the endosome leads to osmotic swelling and finally to endosome rupture. The release is necessary before lysosome mediated digestion of nuleic acids occur (b).[116, 261]

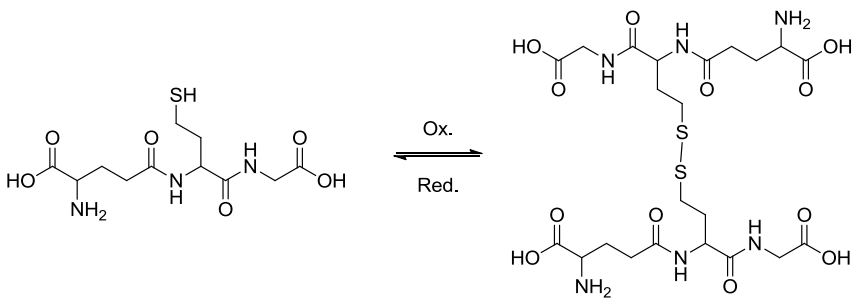
Viruses demonstrate how efficiently nature can modulate membrane barriers since they have evolved sophisticated and specific mechanisms for cell attachment, penetration, and genome replication.[270] A variety of membrane-active natural peptides that mimic recognition, binding, and membrane disruption aspects of the viral proteins have been characterized. Especially those peptides that contain the 20 amino-terminal amino acid sequence of the influenza virus hemagglutinin as well as acidic derivatives are known to be highly membrane active.[270] Upon acidification, the influenza hemagglutinin peptide undergoes conformational changes from a random coil to an amphiphatic alpha-helix exposing hydrophobic domains, which can interact with lipid bilayers and cause membrane disruption.[271]

Synthetic artificial amphiphatic peptides like GALA (repeating units of glutamic acid-alanine-leucine-alanine) or KALA (repeating units of lysine-alanine-leucine-alanine) that mimic natural lytic peptides have been evaluated as well.[271-272]

Diversified molecular structures of different polymers have been designed at nanoscale level. These materials can utilize cellular events like endocytosis and endosomal escape for attaining successful uptake and release of genetic material to the target cell.

1.5.2 Cleavable Carrier Systems

In addition, the ability to escape from the endosome could be accomplished through the incorporation of reducible groups, such as disulfides into the nanocarrier.[209, 273-276] The disulfide bond is a covalent linkage that is obtained from the oxidation of two sulfhydryl groups. The rationale behind this approach is that a redox potential exists between the extracellular and endosomal intracellular space due to the presence of molecules with free sulfhydryl groups such as glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine) and thioredoxin inside the endosome.[209, 277]



Scheme 4. Reversible disulfide formation of GSH.

The intracellular GSH concentration is an additive function of both the oxidized (GSSG) and the reduced (GSH) form, in which the GSSG is recycled to GSH by the enzyme glutathione reductase (Scheme 4).[277]

Another approach to overcome the endosomal membrane called "photochemical internalization" (PCI) has been introduced by Berg et al[278] based upon light activation of a photosensitizer which is specifically located at the membrane of endocytic vesicles which allows the endosomal escape of the polyplexes in a light-inducible manner.[279-283] This novel technology makes use of the generation of singlet oxygen by excitation of a photosensitizer, usually porphyrins or porphyrin-related compounds (Figure 20), which is added together with the non-viral vector to the cell together.[262]

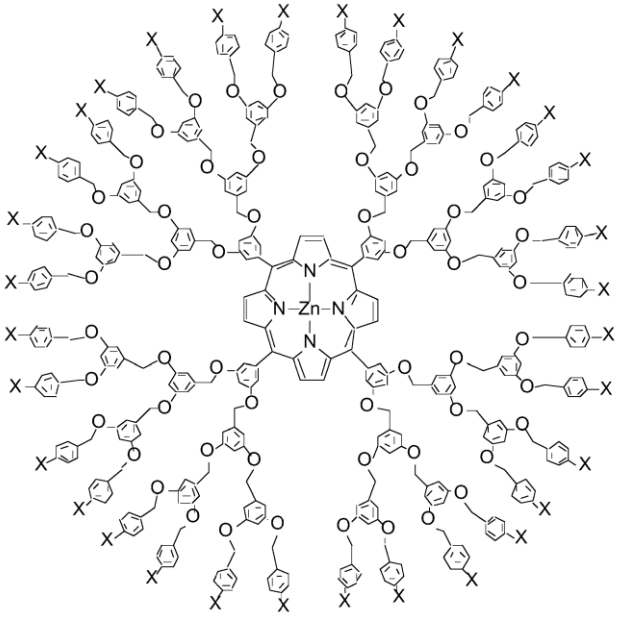


Figure 20. Chemical structure of third-generation aryl ether dendrimer porphyrins dendrimer porphyrins (DPs) with either 32 quaternary ammonium groups (32(+)DPZn, X= $CONH(CH_2)_2N^+Me_3Cl^-$) or 32 carboxylic groups (32(-)DPZn, X= COO^-H^+). Adapted from ref. [284]. Copyright (2003) American Chemical Society.

The photosensitizer binds to the membrane and is internalized via endocytosis.[279] Singlet oxygen is a highly reactive species which is known to oxidize membrane lipids, proteins, amino acids and several other organic macromolecules close to the site of generation.[285]

In the past decade several approaches to deliver DNA/ siRNA into the cell have been developed.[1, 16, 24, 64, 161, 169, 286-288] In general, effective carriers systems require the ability to compact the nucleic acid into particles of virus-like dimensions for cellular internalization, the protection from both extracellular and intracellular nuclease degradation, and finally the intracellular controlled release of the siRNA or DNA-complex.[113] The colloidal surface and

the physicochemical characteristics of DNA/siRNA polyplexes, such as size, charge, hydrophobicity, and buffering capacity, are responsible for controlling the extent and rate of delivery of genes to cells,[289] whereas the efficient release of the gene depends on the characteristics of the transfection vehicles itself.[152] To accomplish an efficient release, the nanocarrier can be developed by following a general schematic design using synthetic polymers introduced by Ringsdorf and co-workers in 1975.[290-292] He proposed that, a number of drug molecules should be bound to a polymeric backbone through a spacer that incorporates a predetermined breaking point to ensure release of the drug from the scaffold after cellular uptake. The system can also contain solubilizing groups or targeting moieties which render the conjugate biorecognizable.[289]

In principle, the gene carrier should be designed with a cleavable linker to trigger the release of the nucleic acid after endocytosis.[128] The release of the complexed gene is essential for achieving an optimal therapeutic benefit and the use of stimuli-responsive nanocarriers offers an interesting opportunity for drug and gene delivery where the delivery system becomes an active participant, rather than passive vehicle, in the optimization of therapy.[293] Such stimuli responsive transfection vehicles can be modified in their ability to compact and release DNA/siRNA near the vicinity of the targeted tissue via environmental stimuli, e.g., the inclusion of photo- and pH-sensitive, redox reactive, and enzymatic, degradable groups (Figure 21).[294]

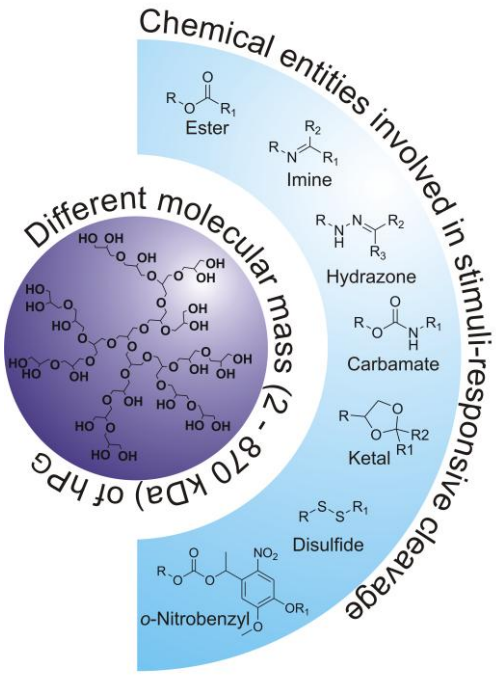


Figure 21. Chemical entities involved in stimuli-responsive cleavage.

Depending on the nature of the interaction between the bioactive molecule and the dendritic polymer, the general concept of triggered release can be divided into two major modes: (i) the release can be triggered by structural changes within the polymeric scaffold (i.e., backbone degradation, cleavage of shell, charging of functional groups, etc.) or (ii) the mechanism of release involves the splitting of the linker between the polymer and the bioactive agent (Figure 22).[293]

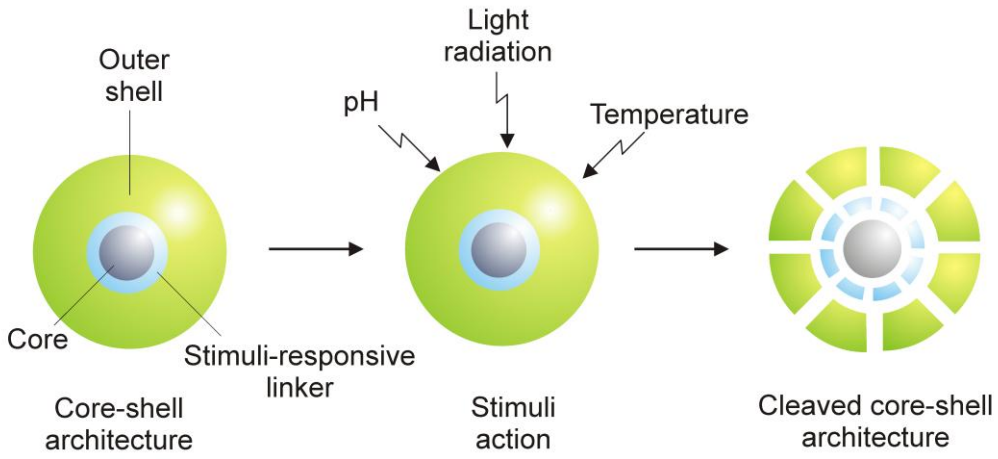


Figure 22. Stimuli-responsive cleavage of nanocarriers. The core-shell architecture consists of a multivalent core, a stimuli-responsive linking unit, and an outer shell. The stimuli-responive linking unit can be designed with various stimuli-responsive entities as illustrated in Figure 21. These core-shell architectures were able to rapidly respond to an stimuli action, e.g., temperature and pH changes or through irradiation with light by inducing structural changes that release the complexed drug/gene.

Since the nucleic acids are not covalently linked to the carrier, only the first mode is of importance for efficient gene therapy.

1.5.2.1 pH-responsive Dendritic Nanocarriers

For stimuli-responsive drug delivery, the application and development of pH-responsive systems has been extensively studied, because of large variations in physiological pH at various body sites in normal as well as pathological conditions such as endosome processing, tumor growth, and inflammation.[275]

The simplest approach for pH-triggered release involves the protonation of certain functional groups at the core or surface of the carrier. Successful transfection efficiency has been promoted by pH-sensitive linkers including ketals,[16] acetals,[295-296] hydrazones, imines,[295] and ester[296] groups. The most studied vectors which exploit the pH gradient are such polycations, such as PEI, polyamidoamine dendrimers, and imidazole containing

polycations that exhibit buffering effects in the endosomal compartment, known as the proton sponge effect.[275, 297] PEI unfortunately displays serious toxic effects and the repeated systemic application as a gene carrier is limited due to the fact that it is non-biodegradable.[298] Therefore, when designing a new carrier to achieve efficient condensation and cytosolic release with low toxicity, the carrier should be degradable. Various efficient polymers bearing hydrolyzable ester bonds have been designed, whereas the degradable linker is either integrated in the polymer backbone or serves as a cross-linking agent.[298] Many research groups generate biodegradable polycations, e.g., by cross-linking nontoxic low molecular weight monomers or oligomers with linkers containing labile bonds.[169] Kloeckner et al. reported the synthesis of biodegradable polycations based on hexanediol diacrylate linked oligoethylenimine (OEI)(Figure 23).[299]

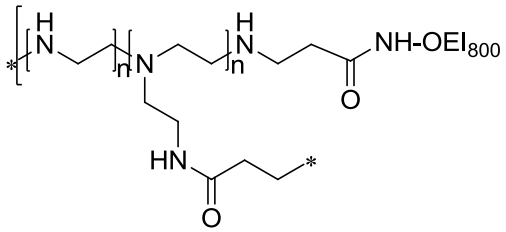


Figure 23. Structure of oligoethylenimine-hexanediol diacrylate. With time, aminolysis of the esters releases the hexanediol moiety from the polymer, resulting in amide-linked derivatives of oligoethylenimine. Adapted from ref. [299]. Copyright (2006) American Chemical Society.

Green et al.[117, 300] reported, e.g., poly(β-amino ester)s as promising materials because they bind and self-assemble with DNA to form stable nanoparticles that effectively enter cells, escape the endosomal compartments, and degrade via hydrolytic cleavage of backbone ester groups.[117] Another approach regarding pH-degradable dendritic architectures has been presented by Wu et al.[301] They have set up an approach with linear poly(amino ester)s for DNA delivery based on the Michael addition polymerization of trifunctional amines and diacrylates which can release the DNA through hydrolyzable ester groups.[301]

1.5.2.2 Light-Responsive Dendritic Nanocarriers

Another alternative for the controlled release of genes is by using light-responsive materials which is advantageous because light is easy to apply, relatively harmless to living organisms, and controllable both spatially and temporally.[16]

Several studies involving photocleavable dendritic architectures have been reported, including self-immolative dendrimers, photoactive surfaces, or photocleavable core based

dendrimers where light of specific wavelengths is used to trigger the release of the encapsulated nucleic acid. Konstiainen et al.[302-303] presented a series of photocleavable dendrons based on spermine chains attached to dendrons through *o*-nitrobenzyl units (Figure 24). The ester linkage to the *o*-nitrobenzyl undergoes photolytic degradation under UV irradiation (λ=350) thus allowing a controlled release of the DNA through the removal of multivalent DNA-binding spermine groups from the surface of the dendrons.[303] Once the spermine groups are cleaved from the surface, they leave behind negatively charged dendrons that will further repel DNA.[303]

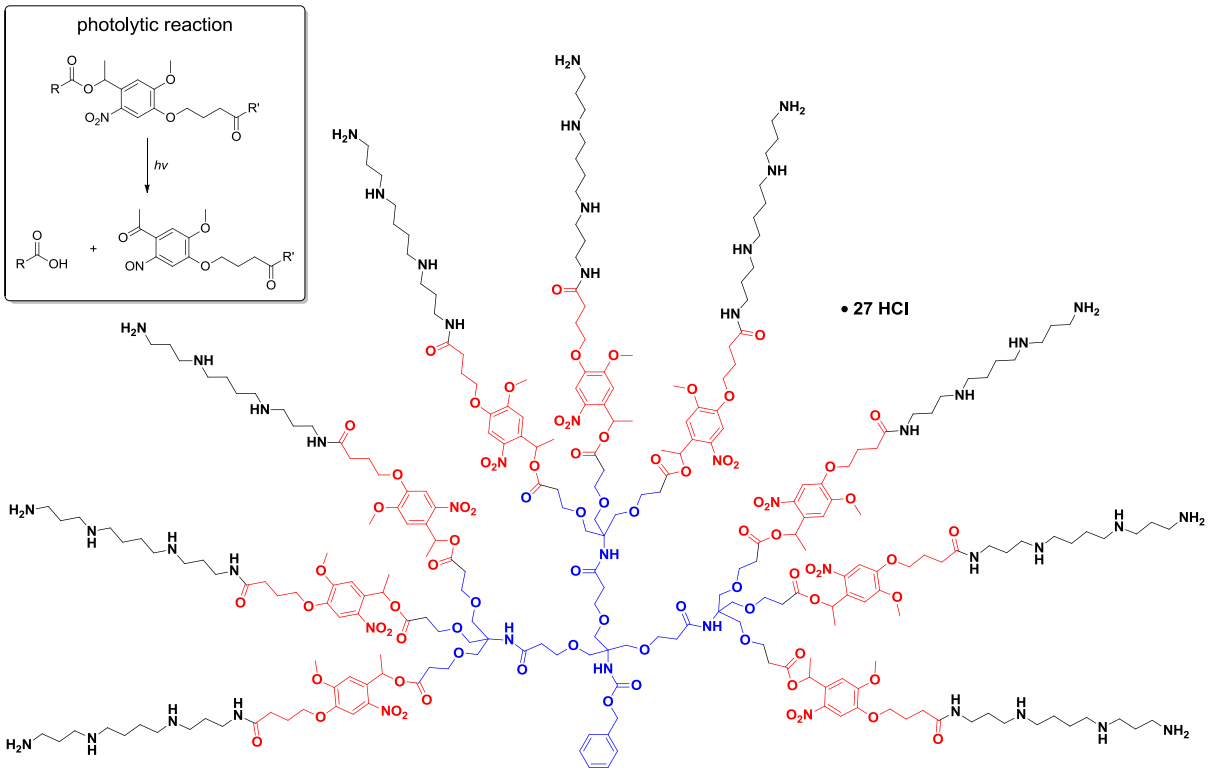


Figure 24. Schematic illustration of a spermine modified photolabile dendron (pIIG2) and its photolytic reaction. Adapted from ref. [303]. Copyright (2007) John Wiley and Sons.

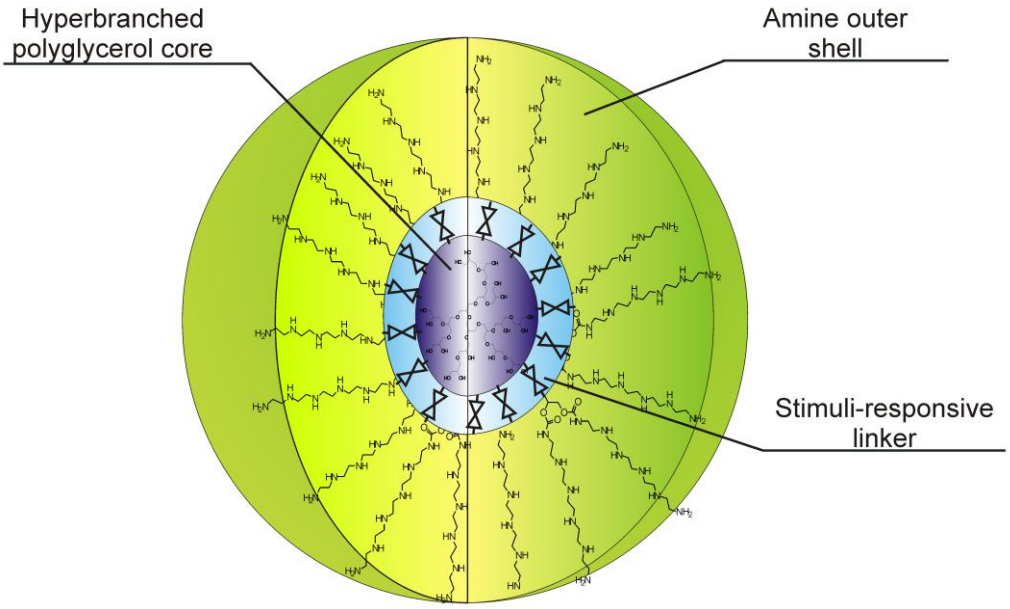
While the transfection efficiency with vehicles, carrying photocleavable linker have shown significant gene transfer, the enhancement of gene expression was accompanied by the photocytotoxicity. However, biologically stimulated release is more favorable since it does not require outside stimulus to induce the cleavage.[16]

Parts of the introduction have been published in *Nucleic Acid Transfection (Top. Curr.Chem.)*, Springer-Verlag Berlin, Heidelberg, 2010, p. 95- 129.

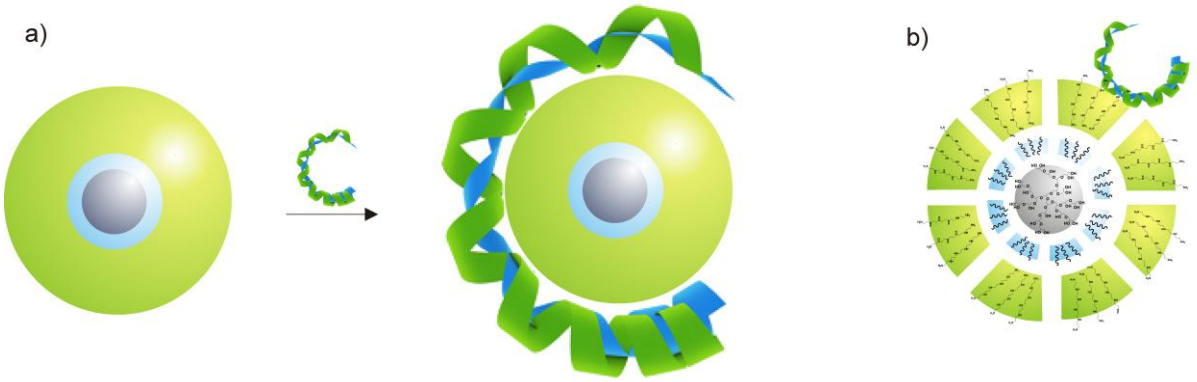
2. Scientific Goals

The general principle of using polymers in gene therapy is based on the formation of a complex between the negatively charged DNA backbone and the positively charged transfection reagent. However, interference of cellular membranes and machineries with positively charged agents results in substantial toxicity. Thus, when designing gene carriers for in vivo applications, care must be taken to keep an optimal balance between toxicity and transfection efficiency. The performance of a non viral gene transfection agent to transfer siRNA into a cell is determined by the capability of the polyamine to complex and compact the siRNA into particles at virus-like dimensions for cellular internalization. Furthermore, the efficiency is dependent on the ability of the carrier to neutralize the negatively charged phosphate backbone of siRNA to prevent charge repulsion at the anionic cell surface and to stabilize the siRNA from both extracellular and intracellular nuclease degradation. In addition, the carrier should lower the lifetime of the fused endosome and lysosome via osmotic pressure and proton sponge effect, and finally they should efficiently decomplex/release the siRNA–complex into the intracellular environment upon specific biological stimuli. The ability to compact the DNA depends on the molecular weight and the degree of branching (DB) of the polymeric carriers. High molecular weight polymers with branched architectures generally exhibit a more globular solution structure and are found to compact siRNA more efficiently. To this end, dendrimers and hyperbranched polymers have found particular application, as partial degradation of PAMAM dendrimers (SuperFect®) was found to be two to three orders of magnitude higher than the stringently synthesized perfect PAMAM dendrimers.[134, 187] A comparison of the resulting DNA complexes formed by each polymer shows that both form compact DNA polyplexes of nearly the same size. Consideration of the above-mentioned cellular and chemical criteria for efficient gene-transfecting agents and relative scarcity of an ideal representative of such carriers in the field of therapeutics motivated us to develop novel highly efficient, stimuli responsive dendritic nanocarriers for siRNA delivery– both for in vitro and in vivo gene delivery.

As hyperbranched polyglycerol (PGs) have already been found to show substantial biocompatibility we have utilized hyperbranched polyglycerol (PG) attached to alkyl amine shells with different numbers of nitrogen atoms to complex nucleotide for in vitro and in vivo applications.[163, 304-312] It is important to mention that the amine shells were attached to the PG core through carbamate linkers to induce pH-responsive cleavability of the shell fragment and consequent decomplexation of the genetic cargo.



The transfection efficiency of a non-viral gene transfection agent to transfer siRNA into a cell is determined by its ability to complex and to decomplex/release siRNA. Therefore, the primary goal of this project will be to design an efficient, biocompatible, and stimuli-responsive nanocarrier with a star-like oligoamine shell. These core-shell architectures fulfil most of the requirements as they can interact well with siRNA/DNA to form nano-scaled polyplexes (a). Their high cationic charge density may facilitate endosomal escape through the proton-sponge effect and the stimuli-responsive shell, either pH- or photodegradable, allows the release of siRNA/DNA (b). Furthermore, these core-shell architectures can be easily modified and therefore easily adapted to the required conditions. Compared to linear polymers, we envision that the dendritic species will exert substantial gene transfection efficiency due to their molecular topology and functional multivalency.



The siRNA is noncovalently bound to the amine shell via electrostatic interactions. The star-like amine shell will be covalently attached through the responsive linker and once the

amine shell will be cleaved from the surface of the dendritic structure, the cationic multivalency effect will be destroyed, thereby leaving just individual amine groups, with only a weaker affinity for siRNA. In this way, the siRNA molecule will be effectively decomplexed upon stimuli responsive degradation.

The transfection efficiency in vitro shall be evaluated and compared with HiPerFect® (a benchmark compound used commonly in gene transfection applications) in HeLaS3 cells. In order to determine the toxicity of the polymers three cell lines with different sensitivities towards polycations will be investigated. Finally protein expression studies shall be performed to evaluate the cellular efficiency of the nanocarriers to cargo genetic materials into the biological environment. Also this approach shall be investigated in vivo.

3. Publications and Manuscripts

3.1 Dendritic Polyglycerols with Oligoamine Shells Show Low Toxicity and High siRNA Transfection Efficiency in Vitro

This chapter was published in the following journal:
W. Fischer, M. Calderón, A. Schulz, I. Andreou, M. Weber, and R. Haag, *Bioconjugate Chem.* **2010**, 21, 1744–1752 (DOI: 10.1021/bc900459n).

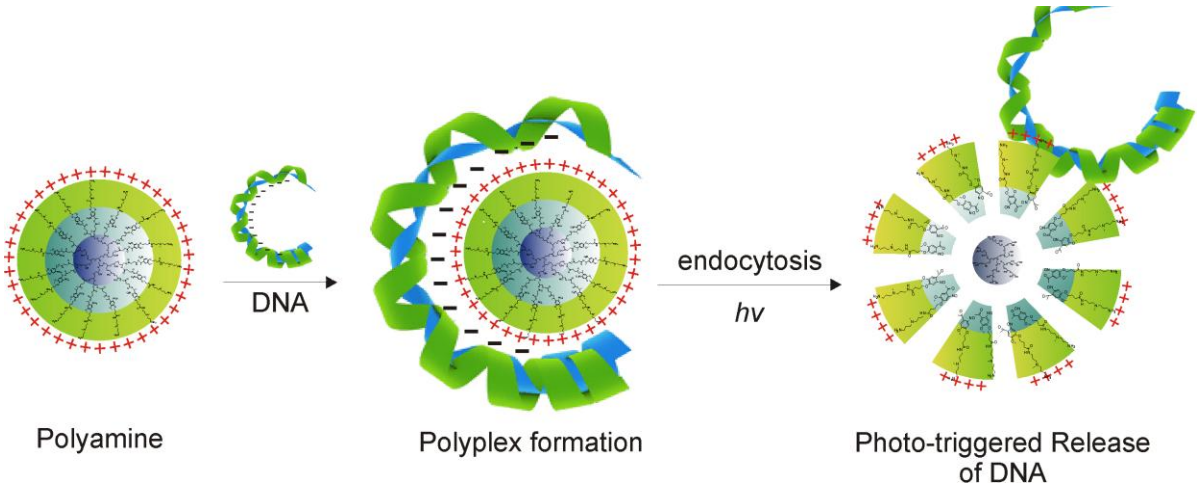
The original article is available at: http://pubs.acs.org/doi/abs/10.1021/bc900459n

3.2 In vivo delivery of small interfering RNA to tumors and their vasculature by novel dendritic nanocarriers

This chapter was published in the following journal:
P. Ofek, W. Fischer, M. Calderón, R. Haag, and R. Satchi-Fainaro, *FASEB* 2010, 24, 3122-3134 (DOI: 10.1096/fj.09-149641).

The original article is available at: http://www.fasebj.org/content/24/9/3122.abstract

3.3 Controlled Release of DNA From Photoresponsive Hyperbranched Polyglycerols with Oligoamine Shells



This chapter was published in the following journal:

W. Fischer, M. A. Quadir, A. Barnard, D. K. Smith, and R. Haag, *Macromolecular Bioscience*, **2011**,11, 1736–1746 (DOI: 10.1002/mabi.201100248).

The original article is available at:

http://onlinelibrary.wiley.com/doi/10.1002/mabi.201100248/abstract

3.4 Hyperbranched Polyamines for Transfection.

This chapter was published in the following journal:
W. Fischer, M. Calderón, and R. Haag, Hyperbranched Polyamines for Transfection in *Nucleic Acid Transfection (Top. Curr.Chem.)*, Springer-Verlag Berlin, Heidelberg, 2010, Volume 296/2010, 95- 129 (DOI: 10.1007/128_2010_64).

The original article is available at:
http://www.springerlink.com/content/576579ht870tx088

4. Summary and Conclusion

Gene therapy involves the transfer of genetic material exogenously into the cell to interfere with cellular machineries for therapeutic or diagnostic purposes.

Cationic polymers are able to effectively condense therapeutic genes to nanosized polyplexes, furthermore they are less cost-expensive and can pack larger quantities of oligonucleotides as compared to biological carrier systems. However, until now they are far less efficient than their viral counter parts, but their efficiency can be increased by adjusting the N/P ratio (the ratio of nitrogen atoms of the cationic polymer to phosphorous atoms of the nucleic acid). On the other hand, increased N/P ratio is associated with an increase in cytotoxicity which limits their clinical application. The aim of this work was to design a novel family of non-viral nanocarriers, which could efficiently transfect and release the siRNA into the cell. Within the framework of this thesis a general and straightforward synthetic concept for preparing pH-sensitive core-shell architectures has been reported, that is based on dendritic polyglycerol. The observation that a partially destroyed dendritic backbone showed even higher transfection efficiencies underlines the significance of using dendritic polyamines with different molecular weights and adjustable flexibility (degrees of branching), both of which influence transfection efficiency and cytotoxicity. By selective modification of the linear and terminal hydroxyl groups of dendritic polyglycerol various core-shell architectures have been created. Attachment of pH-labile shell to the dendritic PG resulted in a polycationic structure, which is able to form stable complexes with siRNA and release the siRNA through cleavage of the carbamate bond. Furthermore, the polymers used for gene delivery should be biodegradable to ensure their long-term tissue compatibility. Several dendritic core-shell architectures have been designed and evaluated, which vary in their molecular weight and differ in the structure of the conjugated amine shell. They were obtained in a two step process. The first step yielded a quantitative formation of phenyl polyglyceryl carbonate, which turned out to be a useful electrophilic PG derivate, while the second step involves the conjugation of the more reactive phenyl polyglyceryl carbonate with various natural and synthetic oligoamines (spermidine, spermine, and pentaethylenhexaamine) to form the biodegradable carbamate bond. In addition, we have synthesized and explored the use of hyperbranched polyglycerolamine, with primary amines in the vicinal 1,2-position.

The ability of the dendritic polyamine to deliver siRNA to the cytosol was evaluated in human HeLaS3 cell line. A series of experiments using different siRNA concentrations (25, 50, and 100 nM) and different amounts of polyamine solutions were conducted to compare

the expression silencing of lamin, CDC2, and MAPK2. The results were compared to the control transfection reagent HiPerFect which is the in vitro benchmark transfection reagent from Qiagen. PG-PEHA and PG-NH_2 were found to be highly efficient, if directed against lamin, and therefore well suited for transfection of HeLaS3 cells. In transfection experiments directed against expression of the proteins CDC2, and MAPK2 only PG-NH_2 were found to be efficient. In order to study the influence of their physiochemical properties over gene transfection, the size, surface charge, hydrophobicity and buffering capacity of the synthesized architectures have been analyzed. Due to their high surface charge under physiological conditions, the nanocarriers were also tested for their cytotoxicity.

Cell viability and red blood cell hemolysis assays were performed to assess their biocompatibility as nanocarriers. First the cytotoxicity of the nanocarriers was evaluated in vitro, exposing U87-Luc cells to serial concentrations of the dendrimers. Viability of cells was assessed 72h later using the XTT reagent. PG-PEHA showed low cytotoxicity values of IC_{50}=30 µg/mL, whereas PG-Amine showed a slightly higher value (IC_{50}=55 µg/mL). A similar range of results was obtained with human neuroblastoma cells. To further evaluate the biocompatibility of the nanocarriers, a red blood cell lysis was performed. The results clearly showed that the nanocarrier were not haemolytic in vitro at concentrations up to 5 mg/mL. Furthermore, the cytotoxicity of the nanocarriers was determined by the enzymatic measurement of lactate dehydrogenase (LDH) amount in the supernatant of the cells cultures.

In both cases, PG-PEHA and PG-NH_2 have shown similar cytotoxic effects compared to the standard transfectant HiPerFect, and since they are only 3-4 % higher than the nontreated controls, it can be considered as low cytotoxic. PG-Amine exhibited the best silencing efficiency versus toxicity ratio in U87-Luc cells. Therefore it was selected for further evaluation and as an in vivo model for its transfection efficiency in a luciferase-tumor model. Significant gene silencing (68 and 85 %) was accomplished in vivo within 24 h after treatment (2.5 or 5 mg/kg luciferase siRNA complexed with 10 or 20 mg/kg PG-Amine, respectively), as measured by photon flux bioluminescence. Low levels of luciferase activity were maintained for three to four days after a single dose of luciferase siRNA complexed with PG-Amine and even for additional three days, after a second dose of the treatment on fourth day.

In another approach, we have synthesized core-shell architectures based on dendritic polyglycerol, in which the shell is linked to the core via a photolabile linker. The synthetic approach was performed in three steps. First, the linear and terminal hydroxy groups were modified using succinic anhydride. The resulting carboxylic acids were activated with pentafluorophenol and subsequently the photolabile linker was added. Finally, by the addition

of different amines, various core-shell architectures were generated. The photo-triggered release of dsDNA (21-mer), as a model for siRNA, was evident from reoccurrence of ethidium bromide fluorescence upon UV-irradiation after preferential quenching and in all cases a complete release was observed after 2 min of irradiation with a 100 W mercury lamp.

In conclusion, dendritic PG-Amine showed enhanced siRNA transfection efficiency and reduced cytotoxicity compared to other dendritic systems examined. Moreover, it could be shown that the siRNA-nanocarrier systems can efficiently inhibit expression of a specific gene in tumor cells. The data strongly implied that PG-Amine is an outstanding candidate for in vivo tumor-directed systemic delivery of siRNA. The biocompatible nanocarriers efficiently assisted double-stranded siRNA transport into tumor tissues and mediated its endosomal release in the cytoplasm of tumor cells.

5. Outlook

The search for an efficient and non-toxic gene transfection vector has led to the design and synthesis of a great variety of dendritic structures. The core-shell architectures introduced in this thesis can be tailored to further enhance their properties. One possibility could be the formation of multi-tasking nanocarriers that are capable of carrying out several functions, particularly the targeted delivery of bioactive gene fragments along to the site of the drug's action. Currently, hyperbranched PG candidates are substantially being considered as delivery enhancers for many bio-active compounds which could substantially increase the internalization of active components specifically into targeted cells thereby enhancing the specific pharmacological activity of the drug molecule and parallel reduction of adverse effects. The potential of such architectures as nanocarriers have been extensively probed, but the use of active targeting as mechanism of gene delivery remains unexplored. Figure 25 shows the concept that needs to be explored in the field of vector engineering by post-modification of hyperbranched polyglycerol, where gene binding site and targeting fragments have been attached concomitantly. Ongoing research along this direction will eventually lead to the discovery of more effective gene delivery vehicles for in vivo applications.

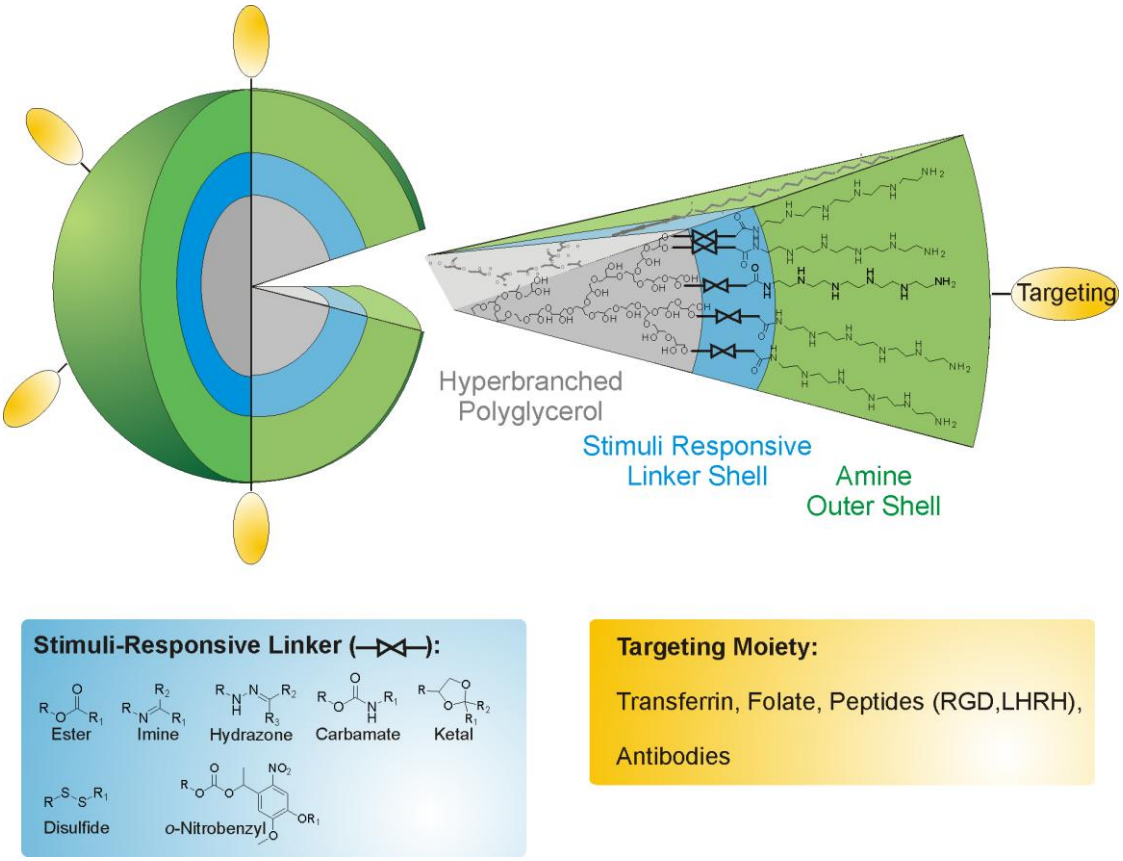


Figure 25. Next generation of gene vectors with targeting moieties for receptor–mediated uptake.

Hyperbranched polyglycerol amine based polyplexes can enter into the cells via an endocytic mechanism. They generally exhibit enhanced cellular uptake with significantly increased gene transfection efficiency. To further improve cell-specific therapeutic efficacy with reducing side effects, cationic polymers are often conjugated with cell recognizable functional moieties for delivery of nucleic acid drugs to the desired target site. In contrast to cationic lipids, a wide variety of structures can be molecularly engineered to have multiple functional moieties in the polymeric carrier. For example, polyethylene glycol (PEG) have been routinely incorporated into cationic polymers for complex stabilization in the blood stream, and various targeting ligands such as folate, peptides (LHRH, RGD), and antibodies are conjugated for cell-specific delivery. In order to enhance the application range of hyperbranched polyglycerol based core-shell architectures a library with different structural variations needs to be constructed which might afford a vector with an optimal transfection/toxicity ratio for in vitro and in vivo delivery of bioactive gene fragments.

6. Kurzzusammenfassung/ Short Summary

6.1 Kurzzusammenfassung

Ein Schlüsselschritt bei der somatischen Gentherapie bleibt das funktionelle Einschleusen der siRNA-Moleküle in das Zytoplasma, da nackte siRNA die hydrophobe Zellmembran nur schlecht passieren kann und körperbedingte Abbaureaktionen deren Wirksamkeit verringert. Ein bedeutender Zugang zu effizienten nicht-viralen Genvektoren beruht auf den multiplen Wechselwirkungen von dendritischen Polyaminen, die die siRNA einerseits ausreichend komplexieren und eine Aufnahme des Polyelektrolyt-Komplexes durch die Zellmembran ermöglichen.

Im Rahmen dieser Arbeit wurde ein einfaches Synthesekonzept zur Herstellung von hochfunktionalisierten, polykationischen Kern-Schale-Architekturen auf Basis von dendritischem Polyglycerin dargestellt. Diese stimulus-reagierenden Polyamine können die siRNA effizient komplexieren, in die Zelle transportieren und nach erfolgter Endozytose aufgrund einer pH-Änderung gezielt im Zytosol freisetzen. Zur Darstellung dieser Architekturen wird der hochverzweigte Polyglycerin-Kern (hPG) mit einem pH-labilen Linker zwischen dem Kern und der hydrophilen Schale, bestehend aus natürlichen oder synthetischen Oligoaminen (z.B. Spermidin, Spermin und Pentaethylenhexaamin), versehen.

Darüber hinaus konnten hyperverzweigte Polyglycerinamine (PG-Amin) mit zahlreichen primären Aminen in der vicinalen 1,2-Position synthetisiert werden.

Durch in vitro Studien in HeLa Zelllinien konnte gezeigt werden, dass alle Polyamine die Expression eines bestimmten Gens hemmen, bzw. signifikant reduzieren können. Ferner konnte in geeigneten Tests die Biokompatibilität aller Nanotransporter bewiesen werden. Die beiden besten Kandidaten aus den in vitro Versuchen wurden anschließend in einem in vivo Modell auf ihre Transfektionseffizienz in einem Luciferase-Tumormodell untersucht. Innerhalb dieses Tests wurde eine signifikantes „Gen-Silencing“ (68 und 85 %), also das Ausschalten eines bestimmten Gens innerhalb von 24 h nach der Behandlung beobachtet. Die Ergebnisse sind vielversprechend und belegen, dass dieser Ansatz auch in vivo großes Potential aufweist.

In einem weiteren Ansatz, wurden photolabile Kern-Schale-Architekturen synthetisiert, die ebenfalls auf dem biokompatiblen hPG-Kern basieren und durch Bestrahlung mit UV-Licht wieder gespalten werden können.

6.2 Short Summary

A key step in somatic gene therapy is the shuttling of functional siRNA molecules in the cytoplasm surmounting systemic degradation and poor cellular transfection of naked siRNA. Dendritic polyamines provide a facile access to efficient non-viral gene delivery vector, where the globular and branched architecture of the carrier scaffold helps to transport the nucleic materials through cell membrane and protect it from cytosolic endosomal/lysosomal compartments.

In this work, a straightforward synthetic approach for generating highly functionalized polycationic core-shell architectures based on dendritic polyglycerol has been reported. These stimuli-responsive polyamines can efficiently transfect and release the siRNA into the cell due to a pH change within the cytosol. The core of these architectures is based on hyperbranched polyglycerol which is attached to the hydrophilic outer shell via a pH-labile linker. The shell consists of natural or synthetic oligoamines, for example, spermidine, spermine, and pentaethylenhexaamine. In addition, hyperbranched polyglycerol amine has also been synthesized, with primary amines in vicinal 1,2-position.

In vitro studies in HeLa cell lines have shown that all polyamines could efficiently inhibit or may significantly reduce the expression of a particular gene. Furthermore, the biocompatibility profile of all nanocarriers has also been established.

The top two candidates from the in vitro experiments were then tested in mice model for their transfection efficiency in a luciferase-tumor model. The results are promising and show that this approach has great potential in vivo (significant gene silencing (68 and 85 %) was accomplished within 24 h after treatment in vivo). In another approach, photo labile core-shell architectures have been synthesized based also on the biocompatible hPG core to generate light-responsive gene-vectors for therapeutic applications.

7. References

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8. Abbreviations

Å	Angström
ADA	adenosine deaminase
AFM	atomic force microscope
CDC2	cell line (cell division control protein 2)
$CHCl_3$	chloroform
D	dendritic unit
Da	Dalton
DB	degree of branching
DNA	deoxyribonucleic acid
dPG	dendritic polyglycerols
DOGS	dioctadecylamido-glycylspermine)
DOSPA	2,3-dioleyloxy-N-[2(sperminecarboxamido)-ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate)
DOTAP	1,2-dioleoyl-3-trimethylammoniumpropane
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl ammonium chloride
EPR	enhanced permeability and retention
EGFR	epidermal growth factor receptor
[G]	dendrimer generation
h	hour
hPG	hyperbranched Polyglycerol
GSH	reduced glutathione
HeLa	cell line (Henrietta Lacks, immortal cell line)
kDa	kilo Dalton
L	linear unit
λ	wavelength
MAPK2	cell line (mitogen-activated kinase 2)
MeOH	methanol
mRNA	messenger RNA

MW	molecular Weight
N/P ratio	ratio of nitrogen versus phosphate
NMR	nuclear magnetic resonance
OH	hydroxyl groups
PAMAM	poly(amido amine)
PCI	photochemical internalization
PD	polydispersity
PEG	poly(ethylene glycol)
PEI	poly(ethylene imine)
PG	polyglycerol
PG-PEHA	polyglycerol pentaethylenhexamine carbamate
PLL	poly-($_L$-lysine)
PPI	poly(propyl imine)
RGD	arginine-glycine-asparagine
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
ROP	ring opening polymerization
SA	succinic anhydride
SCID	severe combined immunodeficiency
siRNA	small interference RNA
T cells	T lymphocytes, group of white blood cells
T	terminal unit
Tf	transferrin
TEM	transmission electron microscopy
ζ	Zetapotential

9. Publications, Patent Application, and Conference Contributions

Publications

1) Paula Ofek, Wiebke Fischer, Marcelo Calderón, Rainer Haag, Ronit Satchi-Fainaro, In vivo delivery of small interfering RNA to tumors and their vasculature by novel dendritic nanocarriers, *FASEB J.* **2010**, 24, 3122-3134

2) Wiebke Fischer, Marcelo Calderón, Andrea Schulz, Ioanna Andreou, Martin Weber, Rainer Haag, Dendritic Polyglycerols with Oligoamine Shells Show Low Toxicity and High Transfection Efficiency in vitro, *Bioconjugate Chem.* **2010,** 21, 1744–1752

3) Wiebke Fischer, Blandine Brissault, Sylvain Prévost, Marta Kopaczynska, Ioanna Andreou, Andrea Janosch Michael Gradzielski, and Rainer Haag, Synthesis of linear polyamines with different amine spacings and their ability to form dsDNA/siRNA complexes suitable for transfection, *Macromol. Biosci.* **2010**, 10, 1073-1083.

4) Wiebke Fischer, Marcelo Calderón, Rainer Haag, Hyperbranched Polyamines for Transfection in *Nucleic Acid Transfection (Top. Curr.Chem.)*, Springer-Verlag Berlin, Heidelberg, 2010, p. 95-129.

5) Sylvain Prévost, Sven Riemer, Wiebke Fischer, Rainer Haag, Christoph Böttcher, Jérémie Gummel, Marie-Sousai Appavou, Michael Gradzielski, Polyplexes of DNA with Polycations — Correlations between Polycation Architecture, Colloidal Stability, Complex Structure and Transfection Efficiency, Biomacromolecules **2011**, 12, 4272−4282.

6) Wiebke Fischer, Mohiuddin A. Quadir, Anna Barnard, David Smith and Rainer Haag, Controlled Release of DNA From Photoresponsive Hyperbranched Polyglycerols with Oligoamine Shells, *Macromol. Biosci.* **2011**, 12, 1736–1746.

Patent Application

R. Haag, W. Fischer, M. A. Quadir, R. Satchi-Fainaro, P. Ofek, Dendritische Polyglycerin-amine für die siRNA-Transfektion, EU-Patentanmeldung (**2008**) EP08156816.4

Conference Contributions

Oral Presentation

1) EuroNanoMedicine, Bled, Slovenien (September 28 -30, 2009)
Oral presentation: SiRNA Transfection by Dendritic Core-Shell Nanocarriers;
Wiebke Fischer, Paula Ofek, Ronit Satchi-Fainaro, Rainer Haag

Poster Presentations

1) Makromolekulares Kolloquium, Freiburg, Germany (23-25 February 2006), participation.

2) International Symposium on Polymer Therapeutics ISPT-07, Berlin, Germany (19-21 February 2007), participation.

3) Makromolekulares Kolloquium, Freiburg, Germany (22-24 February 2007), participation.

4) The 5th International Workshop on Drug Delivery Systems, Prague, Czech Republic (15-18 May 2007)
Poster: New Poly(alkylenimines) for the Transport of SiRNA, Wiebke Fischer, Blandine Brissault, Michael Krämer, Rainer Haag.

4) Polydays 2008, Berlin, Germany, (01-02 October 2008), participation.

5) International Dendrimer Symposium IDS 6, Stockholm, Schweden, (14-18 June 2009).
Poster: SiRNA Transfection by Dendritic Core-Shell Nanocarriers, Wiebke Fischer, Shangjie Xu, Paula Ofek, Ronit Satchi-Fainaro, Rainer Haag.

6) 11th European Symposium on Controlled Drug Delivery, Egmond aan Zee, Netherlands (7-9 April 2010),
Poster: SiRNA Transfection by Dendritic Core-Shell Nanocarriers, Wiebke Fischer, Marcelo Calderón, Paula Ofek, Ronit Satchi-Fainaro, Rainer Haag.

7) Trilateral Symposium on NanoBio Integration, Berlin, Germany (30 September–3 October 2010), participation.

8) Biannual Meeting of the GDCh-Division of Macromolecular Chemistry and Polydays 2010, Berlin, Germany (3-5 October 2010), participation.

10. Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.