



**HAL**  
open science

# Current Designs of Polymeric Platforms Towards the Delivery of Nucleic Acids Inside the Cells with Focus on Polyethylenimine

Fernando A. de Oliveira, Lindomar J.C. Albuquerque, Gwendoline Delecourt, Véronique Bennevault, Philippe Guégan, Fernando C. Giacomelli

## ► To cite this version:

Fernando A. de Oliveira, Lindomar J.C. Albuquerque, Gwendoline Delecourt, Véronique Bennevault, Philippe Guégan, et al.. Current Designs of Polymeric Platforms Towards the Delivery of Nucleic Acids Inside the Cells with Focus on Polyethylenimine. *Current Gene Therapy*, 2021, 21 (5), pp.431-451. 10.2174/1566523221666210705130238 . hal-03524208

**HAL Id: hal-03524208**

<https://hal.sorbonne-universite.fr/hal-03524208v1>

Submitted on 13 Jan 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# **Current Designs of Polymeric Platforms towards the Delivery of Nucleic Acids inside the Cells with Focus on Polyethylenimine**

*Fernando A. de Oliveira,<sup>a</sup> Lindomar J. C. Albuquerque,<sup>a</sup> Gwendoline Delecourt,<sup>b</sup>*

*Véronique Bennevault,<sup>b,c</sup> Philippe Guégan,<sup>b,\*</sup> and Fernando C. Giacomelli <sup>a,b,\*</sup>*

<sup>a</sup> Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Santo André,  
Brazil.

<sup>b</sup> Institut Parisien de Chimie Moléculaire, Equipe Chimie des Polymères, UMR 8232  
CNRS, Sorbonne University, 4 place Jussieu, 75252 Paris Cedex, France

<sup>c</sup> Université d'Evry Val d'Essonne, Université Paris-Saclay, rue du Père Jarlan, 91025  
Evry Cedex, France

\*Corresponding Authors: Dr. Fernando Carlos Giacomelli

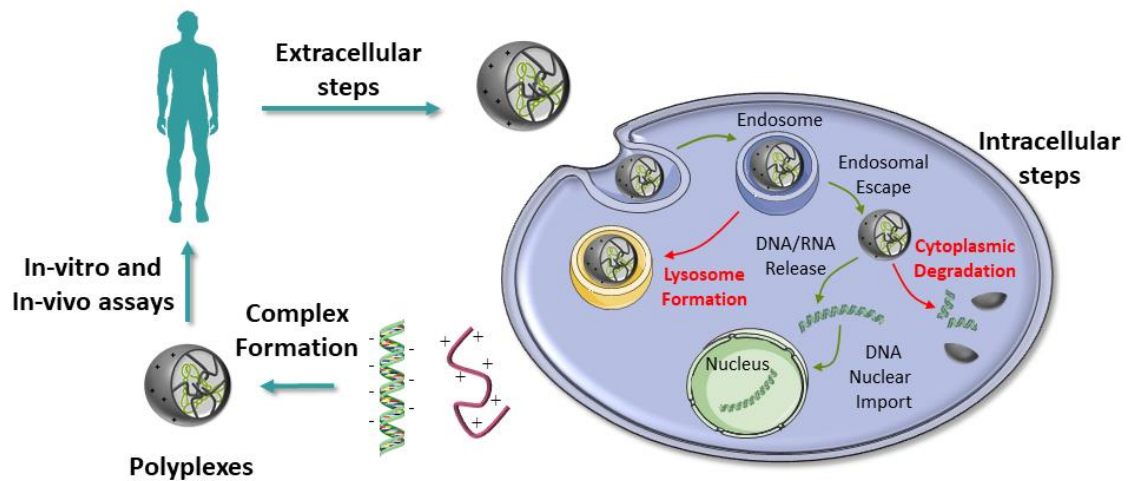
e-mail: [fernando.giacomelli@ufabc.edu.br](mailto:fernando.giacomelli@ufabc.edu.br)

Prof. Dr. Philippe Guégan

e-mail: [philippe.guegan@sorbonne-universite.fr](mailto:philippe.guegan@sorbonne-universite.fr)

# Current Designs of Polymeric Platforms towards the Delivery of Nucleic Acids inside the Cells with Focus on Polyethylenimine

## FOR USE IN THE TABLE OF CONTENTS



we review the gene delivery pathway using non-viral vectors, focusing on the progresses with regard to the use of polyethylenimine and derivatives.

**Keywords:** nanomedicine, gene therapy, gene delivery, non-viral vectors, polymers, polyethylenimine, polyplexes

## **ABSTRACT**

**Background:** Gene delivery is a promising technology for treating diseases linked to abnormal gene expression. Since nucleic acids are the therapeutic entities in such approach, a transfecting vector is required because the macromolecules are not able to efficiently enter the cells by themselves. Viral vectors have been evidenced to be highly effective in this context however, they suffer from fundamental drawbacks, including the ability to stimulate immune responses. The development of synthetic vectors has accordingly emerged as an alternative.

**Objectives:** Gene delivery by using non-viral vectors is a multi-step process that poses many challenges, either regarding the extracellular or intracellular media. We explore the delivery pathway and afterwards, we review the main classes of non-viral gene delivery vectors. We further focus on the progresses concerning polyethylenimine-based polymer-nucleic acid polyplexes, which have emerged as one of the most efficient systems for delivering genetic material inside the cells.

**Discussion:** The complexity of the whole transfection pathway, along with a lack of fundamental understanding, particularly regarding the intracellular trafficking of nucleic acids complexed to non-viral vectors, probably justifies the current (beginning of 2021) limited number of formulations that have progressed to clinical trials. Truly, successful medical developments still require a lot of basic research.

**Conclusion:** Advances in macromolecular chemistry and high-resolution imaging techniques will be useful to understand fundamental aspects towards further optimizations and future applications. More investigations concerning the dynamics, thermodynamics and structural parameters of polyplexes would be valuable since they can be connected to the different levels of transfection efficiency hitherto evidenced.

## 1. INTRODUCTION

Molecular therapy is an encouraging strategy for the treatment of human diseases. Particularly, gene therapy aiming to modify the gene expression in target cells is attractive for treating a variety of malignancies which are linked to the absence, or presence of abnormal genes.[1] The approach can be employed, for instance, in the deactivation of oncogenes, replacement of nonfunctioning tumor suppressor genes, and to transfer genetic materials directly into target cells for permanent changes in their phenotypes. Accordingly, these therapies consider nucleic acids as the active agent and therefore, the active site is inside the cell. The transfer of genetic material into cells can be accomplished essentially by using two approaches: i) in *in vivo* gene editing, the nucleic acids are directly administrated into the body whereas ii) in *ex vivo* gene editing, the cells are previously isolated, edited, and further transplanted into the patient in a more complex procedure.[2] In such an approach, the reverse transfection procedure can also be employed where instead of adding nucleic acids to cells, cells are added to pre-plated nucleic acids.[3] The cellular uptake of naked nucleic acids is nevertheless inefficient due to electrostatic repulsion between their anionic phosphate backbones and the negatively charged plasma membranes.[4] Additionally, whenever nucleic acids are directly administered *in vivo*, they can be rapidly cleared by specific enzymes.[5,6] Accordingly, cargo-delivery platforms are required to provide extracellular protection and stability to the biomacromolecules, allowing further cell internalization and the release of the therapeutic agent in the right intracellular compartment.

In this framework, viral vectors have been formerly demonstrated to be highly effective. Nevertheless, viral delivery systems suffer from significant drawbacks, including the unfavorable interaction with the immune system,[7] besides the production price incompatible with the society financial means, with average costs

sometimes reaching almost \$1 billion per patient.[8,9] These entities can also be oncolytic and mutagenic.[10–12] Hence, strategies to design non-viral gene carriers have been called. The synthetic vectors usually contain cationic units able to bind to negatively charged nucleic acids and drive them towards the intracellular milieu. The polycation polyethylenimine (PEI) and derivatives are recognized as highly efficient gene delivery systems, although they still cannot compete with the cell entry mechanism of viral vectors.[13–15] The supramolecular assemblies based on PEI are however typically linked to significant cytotoxicity due to the membrane disruptive property.[16–18] Truly, cytotoxicity is a common characteristic of many polycations in this regard.

Taking into account the abovementioned considerations, it is currently accepted that more fundamental understanding and optimizations are required. We herein review mainly the recent designs of polyethylenimine-based platforms to deliver nucleic acids inside the cells. Firstly, we explore the delivery pathway of non-viral gene delivery vectors, and afterwards, we briefly underline the main classes of non-viral vectors as well as the main subclasses of polymeric vectors with potential application in gene therapies. We further contextualize the evolution of PEI-based synthetic vectors, and subsequently, we exploit essentially the most recent achievements as well as challenges and opportunities towards enhanced efficacy.

## **2. REQUIREMENTS AND CHALLENGES IN THE DELIVERY PATHWAY**

The potential use of non-viral vectors in gene therapy has been kicked off as justified by the well-known weaknesses of viral counterparts, which are biological entities able to effectively deliver nucleic acids inside the cells at high yields. The most common viral vectors include adenovirus, adeno-associated viruses (AAV), lentiviruses

and retroviruses.[19] While being highly effective as transfecting agents, they are simultaneously associated with fundamental drawbacks, creating the need for synthetic vectors. In this regard, cationic entities that can bind electrostatically to the phosphodiester backbones of nucleic acids to form electrostatic complexes denote a promising class of delivery systems. Such vectors nevertheless must be capable to overcome multiple barriers to successfully delivery genetic material inside the cells,[20] and the nucleic acid binding and condensation is only the first pre-requisite of a non-viral gene delivery vector that can potentially be used in therapies. They must further resist the extracellular barriers, whatever the administration way, and this is particularly critical during intravenous administrations. The serum stability is already one main roadblock because cationic entities are susceptible to unspecific interaction whenever in contact with biological fluids. This may lead to the formation of large aggregates that are rapidly recognized, captured and cleared due to the action of the mononuclear phagocyte system (MPS). The binding of negatively charged proteins (abundant in the bloodstream) may also conduct to unwanted effects such as the activation of serum complement proteins, thus inducing immune responses.[21] The required nucleic acid binding capacity and stability in biological fluids already exclude a non-negligible number of non-viral gene carrier candidates. This issue can be in principle overwhelmed by shielding positive surfaces, for instance, by using neutral elements, therefore reducing the interaction with the naturally negative environments. If these concerns are overcome, then the next step of the journey is the cell adhesion and efficient cellular uptake. The electrostatic complexes are mostly internalized by endocytosis pathways due to their relatively large sizes.[22] The eukaryotic cells commonly uptake nanoparticles in the range 10-300 nm *via* clathrin-mediated endocytosis, whereas caveolae-mediated cellular uptake has a smaller upper limit size around 80-100

nm.[23,24] The polymeric-based gene delivery carriers are most probably internalized *via* both mechanisms (clathrin and caveolae pathways), and the dominant is probably cell line-dependent since both clathrin[25] and caveolae-dependent[26] routes were already suggested to be correlated with higher transfection levels. Besides, other mechanisms such as flotillin-mediated endocytosis have been evidenced.[27] The uptake of nanoparticles *via* clathrin-mediated endocytosis is followed by the pH drop from 7.4 to roughly 6.0 in early endosomes, and 5.0 in late-endosomes and lysosomes. Such an acidic process may lead to the degradation of the assemblies and their molecular bricks, consequently conducting to poor transfection efficiency. The caveolae-dependent route is frequently considered non-acidic and non-digestive, therefore less affected by lysosomal degradation, and possibly leading to higher transfection levels.[28] These considerations are debatable.[29]

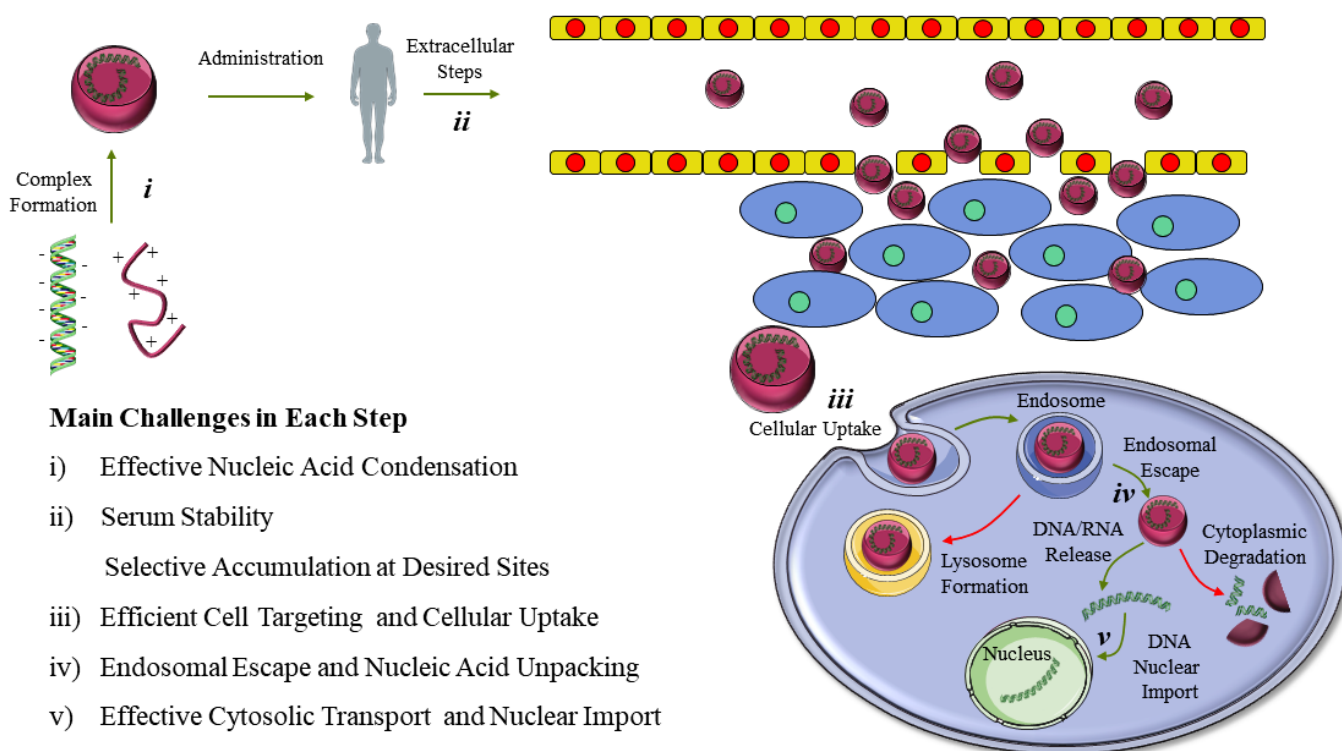
Regardless of the cellular uptake mechanism, the process can be restricted by the presence of shielding agents used to stabilize the supramolecular assemblies in the extracellular milieu. The attachment of specific ligands at their outer surface can then be used as an approach to provide cellular uptake at higher yields as well as targeting capability. This can be particularly useful in tumor treatment since the damaged cells typically overexpress growth factors and receptors that regulate the intracellular concentration of nutrients (transferrin and folate receptors are common examples). Hence, ligands present at the outer surfaces of the assemblies, with high affinity to such receptors, are able to potentialize preferred cell capture. In the step after, the complexes flow towards the intracellular ambient in vesicles named endosomes that contain the engulfed material. The endosomal escape is further required to avoid lysosomal degradation of the therapeutic nucleic acids, which would lead obviously to reduced transfection efficiency. The mechanism used by electrostatic complexes to overcome



the endosomal barrier is still debated, and we discuss the main hypothesis in the following section. Out of the endosomes, the supramolecular assemblies have to be unpacked and, at this stage, instead of extracellular stability, intracellular lability is desired since the dissociation of the complexes is essential to enable the therapeutic activity of the nucleic acids. Taking into account that the nucleic acid condensation is at least partially attributed to electrostatic interactions, the dissociation can be induced by high salt concentrations. The dissociation can be likewise potentialized by the presence of negatively charged polyelectrolytes competing for PEI binding such as observed for PEI/DNA polyplexes in the presence of the polysaccharide heparin.[30] The continuous expansion of linear PEI chains as pH reduces and protonation increases may also occur in endosomal conditions and impact the interaction of PEI with nucleic acids or membranes, thus accounting for the success of PEI as a gene delivery vector.[31] The effectiveness of a gene delivery system also depends on the site of action. While plasmid DNA (pDNA) requires the nuclear import, various RNA molecules (siRNA and miRNA, for example) have the cytosol as the final destination. The CRISPR-Cas9 genome editing tool, which was the subject of the 2020 Nobel Prize in Chemistry, can similarly be used to treat a variety of genetic diseases, and it also requires the nuclei internalization. The search of efficient delivery vectors for the genome-editing agent represents the next challenge in vector design.[32] Truthfully, the cytosolic transport of the therapeutic agent to reach the nuclei membrane is still poorly understood, as it is the mechanism of nuclei internalization, although possibly only small-sized polyplexes (smaller than 50 nm) are allowed to easily enter.[33] This dimension nonetheless diverges from the optimum size (around 90 nm) suggested for efficient siRNA delivery (cytosolic delivery) which balances the circulation time and cellular uptake. Larger

nanoparticles with over 100 nm were demonstrated to be quicker cleared, whereas smaller ones (about 40 nm) were not efficiently uptaken by tumor cells.[34]

Taking into account the abovementioned considerations, it is clear that the gene delivery process by using non-viral vectors is very challenging, thus justifying the fairly limited success so far achieved. One has to face at least with *i*) nucleic acid packaging, *ii*) serum stability; *iii*) cell targeting and cellular uptake, *iv*) endosomal escape and intracellular unpacking and *v*) cytosolic transport and intranuclear delivery. The



schematic representation of the main extracellular and intracellular barriers towards *in vivo* gene delivery using synthetic vectors is cartooned in Figure 1.

**Figure 1.** Schematic representation of the main extracellular and intracellular barriers towards *in vivo* gene delivery using synthetic vectors.

In the next section, we explore in more detail the possible mechanisms enabling the supramolecular systems to escape the endosomes (step *iv*).

### **3. THE DEBATED MECHANISM OF ENDOSOMAL ESCAPE**

Amongst the fairly long list of challenges for the successfulness of non-viral gene delivery vectors, the strategies to overcome the extracellular barriers are overall identified, nevertheless, the pathway used by the supramolecular systems to escape the endosomes is still debated.[35] The step is a key factor determining the transfection efficiency provided by a polymeric material,[36] and it notably attenuates the effectiveness of many cationic carriers. Therefore, more fundamental understanding of molecular biology is required to accurately comprehend the process. Hence, multidisciplinary teams are essential to move forward in the subject. The earliest investigations linked higher transfection efficiency of cationic non-viral gene delivery vectors to the so-called proton sponge effect. The pH-responsive vectors with pKa ~ 7 would enable nucleic acid binding and condensation at pH 7.4, and further protonation in the acidic intracellular environment. The increased ionic concentration inside the endosomes would conduct to an influx of ions (chloride) and water towards the inner cavity of the vesicles, and the osmotic swelling would inevitably lead to the rupture of endosomal membrane releasing the entrapped material into the cytosol. Such a hypothesis nevertheless was not able, for example, to justify the empirical evidences concerning the use of poly(2-methyl-acrylic acid 2-[(2-(dimethylamino)-ethyl)-methyl-amino]-ethyl ester) - PDAMA (the molecular structure of PDAMA is provided in Figure 4). This homopolymer has two tertiary amine groups in each monomeric unit. One was supposed to provide nucleic acid binding and condensation ability at physiological pH, while the other would offer endosomal buffering capacity. The titration of acidified solutions of PDAMA, PDMAEMA and PEI was performed and a

polyelectrolyte behavior was observed for the three polymers, suggesting comparable binding and buffering abilities. Nevertheless, almost negligible *in vitro* transfection activity has been demonstrated for PDAMA/DNA polyplexes without the addition of membrane disruptive peptides. The authors conclude that the proton sponge mechanism cannot be generalized to all the polymers.[37] Additionally, it has been demonstrated that the presence of PEI presumably does not induce changes in lysosomal pH.[38] Besides, realistic calculations suggested that the osmotic pressure generated by the proton sponge effect is not sufficient to solely conduct to the endosome lysis process,[39] and other contributions have to be simultaneously involved. Recently, the direct polyplex-endosomal membrane interaction to explain the endosomal escape has been proposed,[40] and soon after, this has been updated as caused mainly by the interaction of free polycations (not complexed) with the lipid bilayers. The free chains are supposed to be able to interact with anionic phospholipids thereby disrupting the endosomal membrane and assisting the release.[13,41–43] Furthermore, larger molar mass polycations were suggested to interact with membrane proteins, blocking the intervesicular fusion, thus avoiding the trapping in the lysosomes which would result in the degradation of the nucleic acids.[44] Overall, the current knowledge points out that the endosomal escape is possibly mediated by a combination of osmotic-pressure induced membrane rupture as aided by the presence of free polycations, and the last ones also play an important role by interacting with membrane proteins. Nevertheless, there seems not to be a consensus in the scientific community with regard to the main driving force.[45]

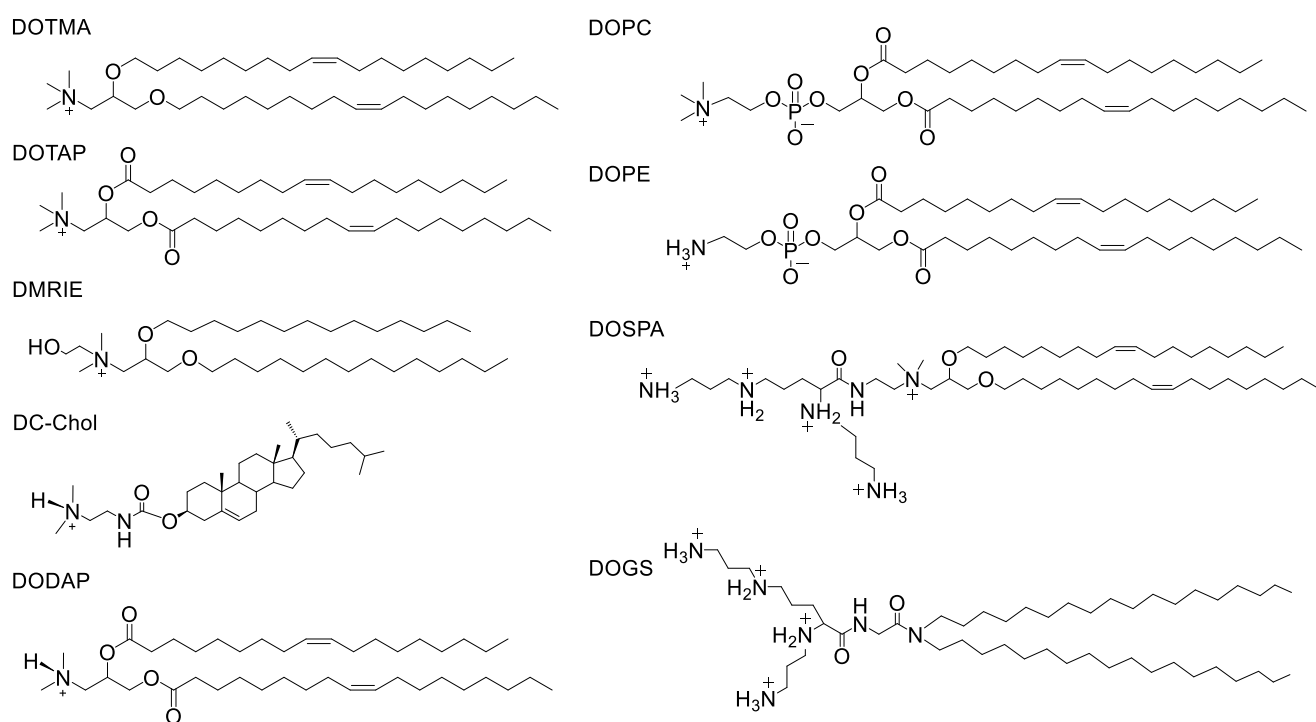
#### **4. MAIN CATEGORIES OF NON-VIRAL VECTORS FOR GENE THERAPY**

The class of non-viral vectors for gene therapy is comprehensive and it includes mainly cationic molecules that can interact electrostatically with the negatively charged phosphate groups of the nucleic acids, therefore leading to the formation of electrostatic complexes. The use of lipids, peptides, lipopeptides and polymers will be briefly reviewed and afterwards, we mainly focus on PEI and its derivatives.

#### **4.1. Lipids**

The supramolecular structures produced by the complexation of lipids and nucleic acids are named lipoplexes, and the transfection procedure is defined as lipofection. The lipids used as transfecting agents are composed by a hydrophilic, and commonly cationic head, linked to a hydrophobic domain. The amphiphilic feature of such molecules enables their self-assembly into different morphologies depending on the packing parameter (vesicles and micelles, for instance). Typically, vesicles (named liposomes) are used in the field of gene delivery. They interact *via* electrostatic forces with nucleic acids producing different hierarchical structures. The lamellar and hexagonal arrangements are composed by an alternation of lipid and nucleic acid domains. The liposomes composed by phosphatidylserine were demonstrated to efficiently deliver DNA into cells[46] and later, high transfection rates were also reached by using the monovalent cationic lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTMA),[47] 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP)[48] and dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium (DMRIE).[49] Additionally, lipids having a neutral head, for instance, dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC), have been investigated as “helpers” in combination with cationic counterparts.[50,51] Besides permanently positively charged cationic lipids, examples of ionizable

monovalent and multivalent lipids used towards the same goals include 1,2-dioleoyl-3-dimethylammonium propane (DODAP),[52] dioctadecylamido-glycylspermine (DOGS),[53] 2,3-dioleoyloxy-N-[2(spermi-necarboxamido)ethyl]-N,N-dimethyl-1-propanaminium tri-fluoroacetate (DOSPA)[54] and cholesterol derivatives.[55,56] Figure 2 provides the molecular structure of the main lipids used in the formulation of non-viral gene delivery vectors. The class of lipid-based DNA therapeutics has been recently reviewed,[57] also with particular focus on the treatment of gliomas.[58]



**Figure 2.** Molecular structure of main lipids used in non-viral gene delivery formulations (ionizable lipids are displayed as protonated species).

Concerning the cellular uptake and intracellular pathway, lipoplexes are internalized presumably by endocytosis, and the endosomal escape occurs *via* the so-called flip-flop mechanism. The lipid-nucleic acid complexes are able to destabilize the endosomal membranes inducing a flip-flop of anionic lipids from the cytoplasmic-

facing monolayer which diffuse into the complex and form a charge neutral ion pair with the positively charged lipids. The nucleic acid-cationic lipid interaction is weakened and ultimately, the therapeutic biomacromolecule is released into the cytoplasm.[59] Apart from the sufficient transfection levels, the ability of lipids to disrupt the cellular membrane may lead to off-target effects and cytotoxicity depending on the chemical nature and concentration.[60]

## **4.2. Amino Acids and Peptides**

The main amino acids used in the intracellular delivery of nucleic acids include lysine (Lys, K), arginine (Arg, R) and histidine (His, H). Typically, poly(amino acid)s are used for such purpose as hereafter discussed in more details. The use of peptide-based nanocarriers has been encouraged due to the possibility of precisely control the syntheses steps, therefore building desired sequences, as well as incorporating targeting ligands. The introduction of solid-phase peptide synthesis (SPPS) by Merrifield was a milestone in this regard.[61] The molecules are bound to a solid support (resin), and the synthesis takes place step-by-step using protecting group chemistry. The intermediate peptides can be eliminated by washing and filtration. In this category of non-viral vectors for gene delivery, the cell penetrating peptides (CPPs) are also evoked.[62,63] These are chains consisting of 4 to 40 highly charged amino acids (Arg and Lys, for example) or sequences with hydrophilic and hydrophobic domains (amphipathic molecules).[64] They have the ability to translocate the plasma membrane and can be used in a variety of biomedical applications.[65] The classical example of CPP is the TAT sequence which is rich in positively charged Arg and Lys residues.[66] The protein penetratin[67] is another example of CPP that has been used to optimize the

intracellular delivery of nucleic acids. Penetratin was demonstrated to notably augment the transfection yields of polymethacrylate-based non-viral gene delivery systems.[68]

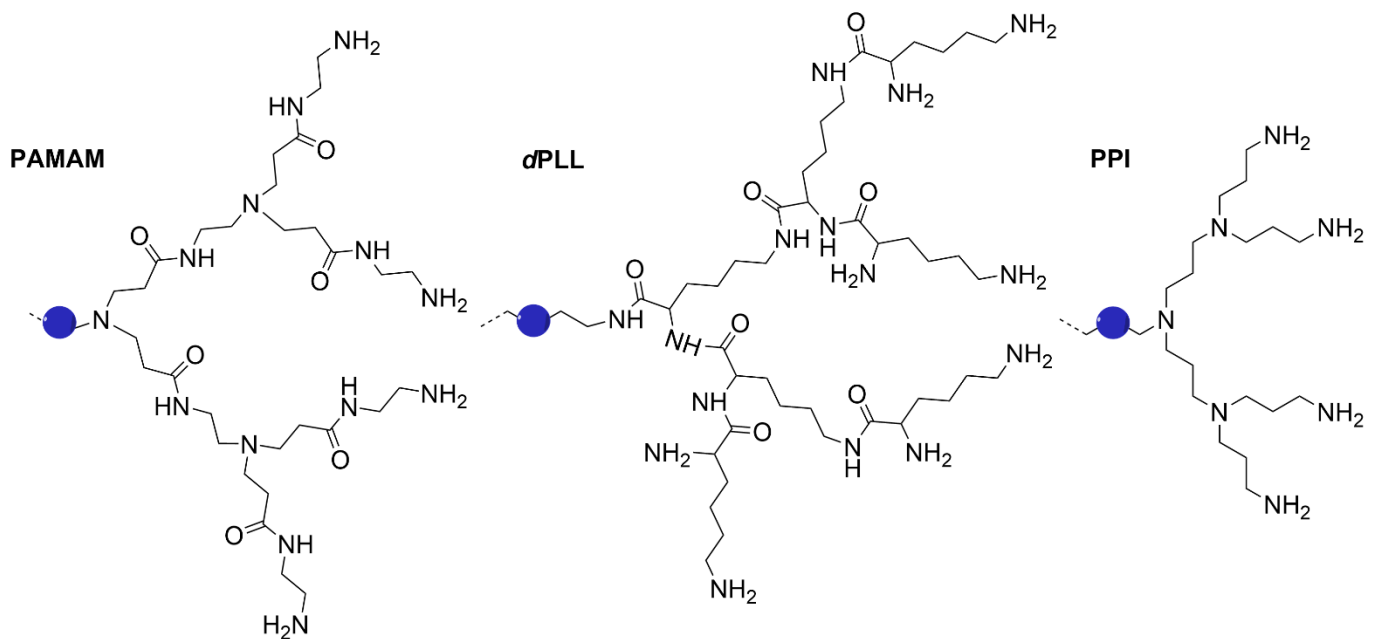
### **4.3. Lipopeptides**

The fatty acids can be coupled to sequence-defined-peptide chains by SPPS to produce lipopeptides. The use of lipopeptides in nucleic acid delivery has been explored by Wagner *et al.* for instance using artificial units such as succinoyl tetraethylene pentamine (Stp) and succinoyl pentaethylene hexamine (Sph).[69,70] The building blocks were combined with cationic amino acids (such as lysine) and fatty acids. The entities were demonstrated to be useful in the delivery of pDNA, siRNA, mRNA and antisense oligonucleotides.[70–75] The importance of tyrosine trimers to improve the serum stability and buffer capacity of manufactured sequences, as well as enhanced effectiveness by using cysteine residues, have been also highlighted.[72,76] Sequence-defined pH-sensitive lipopeptides containing a cationic head, amino acid residues and lipophilic tails were equally investigated.[77,78] These arrangements were able to condense siRNA into compact nanoparticles with low cytotoxicity, and provided high transfection yields.

### **4.4. Dendrimers**

Dendrimers are hyperbranched structures with globular morphology and, whenever they have a cationic character, they can bind to nucleic acids thereby forming the so-called dendriplexes. The dendrimer poly(amido amine) (PAMAM)[79] has been widely investigated in this framework. PAMAM is only moderately protonated at physiological pH while highly protonated at acidic environments. Other non-viral gene





delivery vectors belonging to the same class include dendritic polylysine (*dPLL*)[80] and polypropylenimine (PPI).[81,82] The molecular structures of these dendrimers are provided in Figure 3.

**Figure 3.** Molecular structure of the main dendrimers used in non-viral gene delivery formulations.

The polypropylenimine was demonstrated to hold a relevant degree of cytotoxicity, which can nevertheless be circumvented by reducing its molar mass with a balanced effect in the transfection rate.[83] The dendritic PLL is relevant in this class of non-viral gene delivery vectors. Indeed, the architecture was reported to have a notable influence on the transfection rate of poly(amino acid)s, and higher yields were demonstrated for *dPLL* compared to the linear configuration.[84] The evidences were linked to the lower pK<sub>a</sub> of the globular architecture, resulting in weakened dendrimer-gene interaction, thus allowing an easier intracellular nucleic acid unpacking. Besides, one can still find macromolecules based on dendritic carbosilane[85] and

glycodendrimers[86] evaluated as potential gene delivery vectors. Nevertheless, they are normally effective only when conjugated with positively charged segments.[87]

#### **4.5. Polymers**

Cationic polymers (polycations) can bind *via* electrostatic forces to nucleic acids producing supramolecular aggregates with overall dimensions comparable to those of viruses. The phosphate groups of the nucleic acids normally interact with positively charged synthetic vectors leading to the formation of electrostatic complexes (named polyplexes). Amongst the subclasses of polymers that can be used in nucleic acid delivery, the most important are nitrogen-based polycations including polypeptides, polymethacrylates, polysaccharides, poly( $\beta$ -amino esters), polyvinylamines and polyamines (mostly, polyethylenimine and derivatives). The last one is indeed the main focus of this review nevertheless, we briefly describe other subclasses of nitrogen-based polycations investigated towards the same goal in the following section.

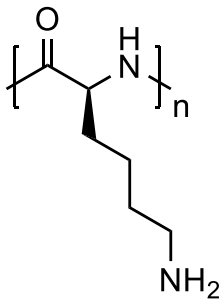
### **5. MAIN CLASSES OF POLYMER-BASED NON-VIRAL VECTORS FOR GENE THERAPY**

#### **5.1. Poly(amino acid)s**

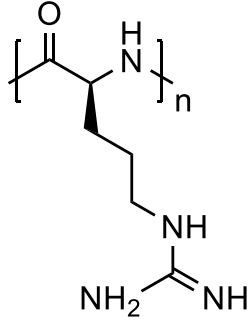
Regarding the class of poly(amino acid)s, poly-L-lysine (PLL) is certainly the most commonly found in the literature. The polymer can be synthesized by ring-opening polymerization of  $\epsilon$ -benzyloxycarbonyl-L-lysine N-carboxyanhydride (Lys(Z)-NCA).[88] The polyplexes produced by using PLL are efficiently uptaken by cells however, they suffer of a serious disadvantage which is the poor ability to escape the endosomes due to the low buffering capacity of PLL below physiological pH ( $pK_a \sim$

10.5),[89] thereby resulting in reduced transfection efficiency. This could be to some extent circumvented by grafting histidine or poly-L-histidine (PLH) into the main chain, then providing buffering capacity due to the histidine protonation at  $\text{pH} < 6$ . [90] Poly-L-histidine holds buffering capacity, but poor nucleic acid binding capacity at physiological pH due to insufficient cationization. The poly(amino acid)s poly-L-ornithine (PLO)[91] and poly-L-arginine (PLR)[92] are also considered as gene carriers. Indeed, polyplexes produced using PLO were evidenced to provide overall better results compared to those manufactured using PLL as attributed to the greater affinity of PLO to pDNA, therefore condensing the biomacromolecule at lower N/P ratios.[93] The use of PLR is useful particularly towards enhanced cellular uptake as inspired by cell-penetrating peptides. Although a couple of poly(amino acid)s demonstrated reasonable transfection rates, the polyplexes suffer of a common drawback which is the high level of cytotoxicity, particularly when the molar mass is elevated (this is frequently evidenced for polycations as previously stated). The coating by using poly(ethylene glycol) (PEG), such as in the construction of the gene carrier CK<sub>30</sub>PEG10K, attenuates cytotoxicity. This macromolecule consists of a 30 mer PLL conjugated to 10 kDa PEG. Electrostatic complexes produced using CK<sub>30</sub>PEG10K were demonstrated to be non-immunogenic, able to condense large DNA chains and efficiently deliver them into RPE cells, therefore with potential application in ocular gene therapies.[94–96] Figure 4 provides the molecular structure of the main polymers investigated as potential gene delivery vectors, including the class of poly(amino acid)s.

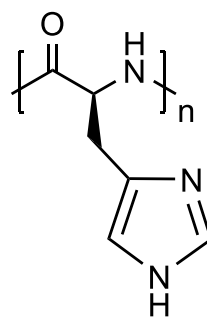
PLL



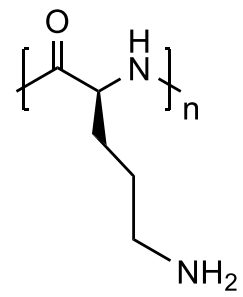
PLR



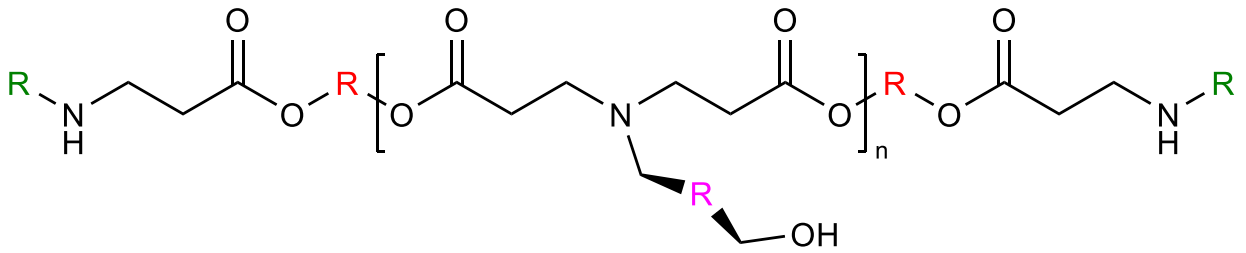
PLH



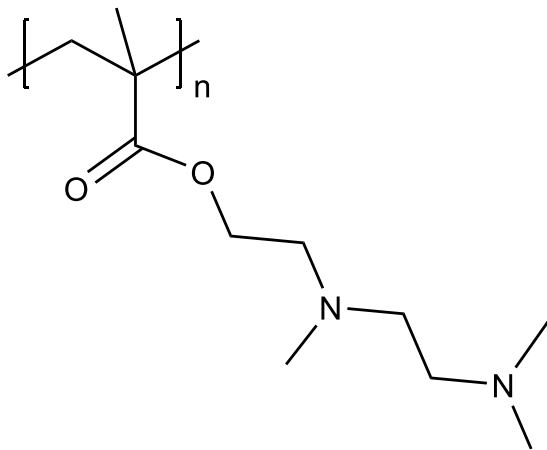
PLO



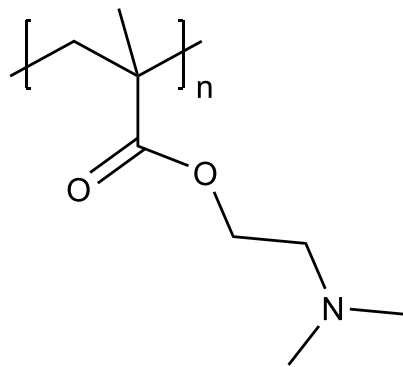
PβAE



PDAMA

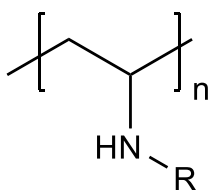


PDMAEMA

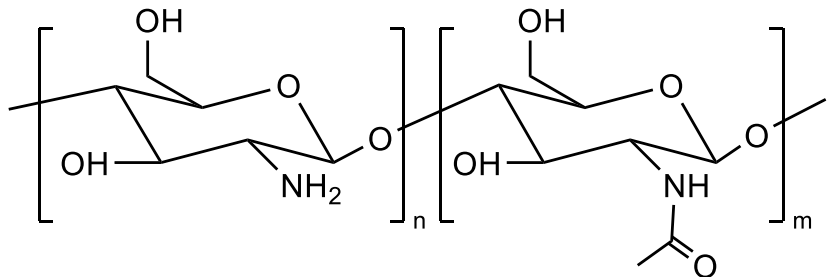


PVAm / PMVAm

R = -H    R = -CH<sub>3</sub>



Chitosan / Chitin



**Figure 4.** Molecular structure of the main polymers used in the manufacturing of non-viral gene delivery formulations.

## 5.2. Polymethacrylates

Polymethacrylates containing amino groups in their structure have been evaluated as gene delivery vectors. These polymer-based carriers are commonly synthesized *via* controlled polymerization techniques, thus enabling the synthesis of well-defined chains. The investigations are essentially centered on poly[2-(dimethylamino) ethyl methacrylate] (PDMAEMA) which contains a tertiary amino group and can be found in different architectures (linear and star-shaped, for instance). Although polyplexes produced from PDMAEMA can be internalized efficiently by cells, the endosomal escape has been demonstrated to be the main bottleneck for its effectiveness.[97,98] The transfection efficiency is notably influenced by the molar mass[97] and the architecture.[99] Beneficial synergic effect in this regard was reached by using a mixture of PDMAEMA and poly( $\beta$ -amino ester) (this class of potential non-viral gene delivery vectors is discussed hereafter).[100] Furthermore, the transfection efficiency of polymethacrylate-based polyplexes has been recently demonstrated to be enhanced by using higher contents of primary amino groups in the chains, whereas higher amounts of tertiary amino groups hamper the process.[101] The authors additionally highlighted that the endosomal escape is likely to be related to a strong polymer-membrane interaction rather than the popular proton sponge effect. In this category, we recall the attempts concerning PDAMA,[37] as the reported results were important to deepen the discussions with respect to the actual mechanism of endosomal escape.

### **5.3. Poly( $\beta$ -amino ester)s**

Poly( $\beta$ -amino ester)s are polymers with properties of tertiary amines and esters, therefore presumably able to bind to nucleic acids at physiological pH, and holding degradable and pH-responsive properties. They are typically synthesized by Michael addition from a diacrylate and an amine. Accordingly, the large portfolio of amine and diacrylate monomers as well as post-polymerization approaches enable the manufacturing of large polymer libraries with different properties concerning pH responsiveness and degradability. The linear form of poly( $\beta$ -amino ester)s (P $\beta$ AEs) was introduced by Langer *et al.*, [102] and it has been demonstrated that the chemical nature of the terminal groups significantly impacts cytotoxicity and transfection rates. [103,104] This feature encouraged the synthesis of branched P $\beta$ AEs, therefore with a higher number of available terminal groups permitting further optimizations. [105,106] The polymer structure, especially the degree of hydrophobicity, influences the degradation and DNA condensation ability in this class of potential non-viral gene delivery vectors. [107] Truly, the history of P $\beta$ AEs in gene delivery is still fairly recent, and there is no a clear scenario concerning clinical trials at the moment. The synthesis, formulation and biomedical applications of P $\beta$ AEs have been recently reviewed, [108] as well as the use in gene delivery formulations. [109]

### **5.4. Polysaccharides**

The polysaccharides overall do not hold cationic charges nevertheless, one exception is chitosan. This justifies the fairly high number of gene delivery investigations based on such natural polymer. [110] This polysaccharide is composed by  $\beta$ -(1 $\rightarrow$ 4)-linked D-glucosamine and *N*-acetyl-D-glucosamine as depicted in Figure 4.

Chitosan is the deacetylated form of chitin which is obtained by enzymatic deacetylation or chemical hydrolysis.[111] The DNA condensation is promoted, as usual, by the interaction of the phosphate groups of nucleic acids and the amino groups of the natural polymer. The ability of chitosan to provide gene transfection is nevertheless critically affected by the environmental pH.[112] These authors moreover highlighted that the particle size does not notably influence gene knockdown, nevertheless, the behavior is markedly influenced by the particle charge and interaction with organic matter. Indeed, at least to some extent due to its  $pK_a \sim 6.5$ , [113] chitosan itself has a fairly limited solubility as well as limited nucleic acid binding capacity at physiological pH (7.4). Nevertheless, the functional amine groups can be used as active sites, and chemical modifications are relatively easy to be accomplished. Hence, a variety of attempts have been conducted towards improving its performance. The transfection rates of chitosan-based polyplexes depend notably on the degree of deacetylation. Such a parameter must be precisely selected in order to compromise transfection levels and the formation of stable complexes.[114,115] Besides the fairly common investigations concerning chitosan, and particularly its derivatives (such as in combination with PEI, for instance), [116] a number of other investigations dealing with different polysaccharides including dextran and cyclodextrin (CD) can be found in the literature. Nevertheless, these natural polymers poorly interact with nucleic acids and they are typically found as segments of hybrid gene delivery vectors, generally grafted to nitrogen-based cations or polycations. Cyclodextrin-based nucleic acid delivery systems has been recently reviewed, [117] and dextran-spermine, [118] dextran-PEI, [119] dextran-histidine [120] and dextran-chitosan [121] were also investigated.

## **5.5 Polyvinylamines**

The polyvinylamines (PVAm) are obtained generally by controlled hydrolysis of polyvinylamides previously prepared by radical polymerization[122] and recently, progresses in the polymerization protocols allowed for well-defined polyvinylamine-based polymers with controlled molar masses.[123] Polyvinylamine and poly(*N*-methylvinylamine), respectively bearing primary and secondary amines, demonstrated high transfection yields and low levels of cytotoxicity.[124] The effectiveness of polyvinylamines with secondary amines is molar mass-independent, whereas remarkable influence of the molar mass was observed for the case of primary amines. Furthermore, it has been demonstrated beneficial effects potentialized by the incorporation of imidazole and guanidine groups in the polymer chains.[125] The efficacy is nonetheless dependent on the chemical modifications performed, particularly the degree of substitution, and transfection levels compared to PEI in a variety of cell lines can be reached by playing with such parameter. Although an old family of cationic entities, this class of polymers has been seldom explored in the framework of gene delivery, and further advances are expected to be shown shortly.

## **5.6. Polyethylenimine**

Concerning polymeric gene vectors, certainly the most investigated polymer is polyethylenimine (PEI) in different architectures and derivatives. This is justified by the high levels of transfection activity evidenced, although frequently linked to high degrees of cytotoxicity. Accordingly, strategies for optimizing its performance and overcome such drawback are still a field of active research. The PEI chains are easily substituted by using a variety of different chemical groups, and this feature encouraged the manufacturing of different derivatives. We review the main approaches towards overcoming different extracellular and intracellular barriers in the following section.



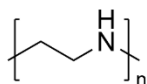
## **6. POLYETHYLENIMINE-BASED NON-VIRAL VECTORS FOR GENE THERAPY**

### **6.1. The Discovery of PEI as a Potential Non-Viral Gene Delivery Vector**

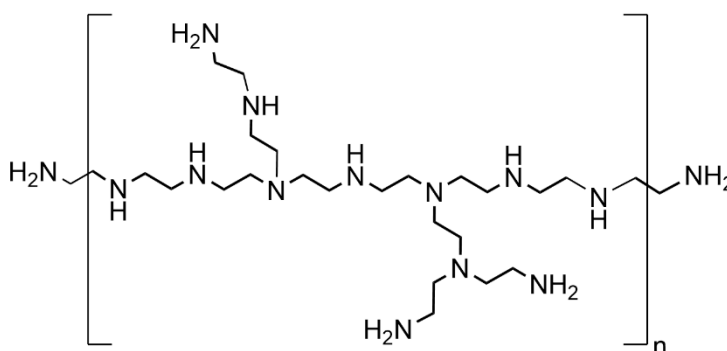
The PEI-based polycations are considered as a second generation of polymers investigated as potential gene delivery vectors.[126] The second-generation embraces PEI and the already mentioned dendrimer PAMAM. The amine groups in the PEI chains are only partially protonated, and there are many of them not protonated at physiological pH, which are capable of picking up more protons when pH reduces.[127] This is a notable difference compared to polycations of the first generation (PLL, for instance) which is fully protonated at physiological pH, therefore with limited endolysosomal buffering capacity, and then requiring the addition of lysosomotropic additives or membrane disruptive agents to promote the endosomal escape. Indeed, PEI has started to be vastly investigated towards the intracellular delivery of nucleic acids precisely due to the sufficient residual positive charges at physiological pH enabling its binding to nucleic acids, besides the buffering capacity at lower pH. The PEI chains are fairly flexible in the chemical point of view and then, their features can be tuned to some extent to optimize polyplex stability, cytotoxicity, cellular uptake and transfection efficiency. Nevertheless, although PEI is able to mediate endosomal escape of polyplexes and further gene transfection, the efficiency is only moderate compared to

viruses. Truly, the PEI discovery allowed a deeper understanding of the gene delivery pathway along with a list of requirements for the successfulness of non-viral gene delivery vectors based on polymeric materials. We underline in the following sections essentially PEI derivatizations hypothesized to optimize principally the cytotoxicity-gene transfection balance.

(A) Linear PEI



(B) Branched PEI



## 6.2. Main Architectures of PEI Chains

The cationic polymer PEI is mostly found in two different architectures as depicted in Figure 5. This depends essentially on the synthetic strategy used to manufacture the material.

**Figure 5.** Chemical structures of linear (A) and branched (B) PEI.

The linear (*l*PEI) chains are synthesized typically *via* the cationic ring-opening polymerization of 2-ethyl-2-oxazoline (PEtOx) with further hydrolysis, whereas the acid-catalyzed polymerization of aziridine leads to the manufacturing of branched chains (*b*PEI). These are the configurations more commonly used to deliver nucleic acids inside the cells, although other topologies were investigated, such as comb-

like[128] and cyclic chains.[129] The PEI chains contain a very high density of nitrogen atoms which allows chain protonation, particularly in acidic ambients. The branched configuration (*b*PEI) contains primary, secondary and tertiary amines usually in the ratio 1:2:1 and with different pKa (4.5 for primary, 6.7 for secondary and 11.6 for tertiary amines).[130] The presence of tertiary amines presumably provides a high density of positive charge at physiological pH, thus allowing nucleic acid binding. We nevertheless highlight that these pKa values cannot be easily assigned due to the effect of local groups and polyelectrolyte behavior. Other numbers have been also suggested, such as the predominant protonation of primary[131] or secondary[132] amines at neutral pH. The degree of protonation anyway increases by reducing the pH. The linear configuration has only secondary amines and empirical data as well as theoretical calculations suggest that roughly 60% of the amino groups are protonated at nearly neutral pH, regardless of the chain architecture (linear or branched).[133,134] This feature permits both architectures (*l*PEI and *b*PEI) to condense genetic material, and they mediate endosomal escape and gene transfection. The linear configuration has been reported to be more efficient for gene delivery purposes as attributed to the higher stability of *b*PEI polyplexes, leading then to less efficient unpacking of the biomacromolecules in the intracellular milieu.[135]

### **6.3. Strategies to Enhance Effectiveness**

The research concerning PEI-based polyplexes is highly active and the more we progress the more we understand the main challenges which still limit clinical outputs. The transfection efficiency and cytotoxicity of the polymeric gene delivery vectors are usually linked to the chain's molar mass. This is indeed a nasty correlation since usually the longer the chains, the higher the transfection rates, but higher levels of cytotoxicity

are observed at the same time. Accordingly, endless efforts have been already dedicated to target more efficient polymeric vectors of lower cytotoxicity. The long way from the synthesis of a non-viral gene vector towards the delivery of the nucleic acid inside the cells is highly complex as underlined previously. Different steps of the delivery pathway require commonly conflicting properties on the chemical and structural points of view. Therefore, the requirements of non-viral vectors throughout the whole pathway (nucleic acid binding and condensation, serum stability, cell targeting and cellular uptake, efficient endosomal escape, intracellular unpacking, cytosolic migration and nuclei entry) have to be precisely understood to hypothesize chemical modifications able to optimize the delivery performance. The PEI chains can be manufactured by using different synthetic strategies depending on the desired configuration, and derivatives can be constructed during the polymerization procedures, or by using post-polymerization protocols.

### ***6.3.1. Synthetic Approaches towards the Manufacturing of PEI Derivatives***

The chemical modification of PEI chains is expected to optimize the properties of the non-viral gene delivery vectors. This can be theorized aiming a variety of goals within the delivery pathway. The functionalization with targeting ligands is a universal strategy in nanomedicine to target specific cells. The modifications are also useful to improve stealth properties and endosomal escape capacity, to enable the intracellular unpacking of the genetic material, as well as to reduce cytotoxicity. In this context, different strategies can be used to modify the chains. The synthesis of *l*PEI is carried out usually *via* the acidic or basic hydrolysis of poly(2-oxazoline)s. Functional groups can be introduced during the oxazoline polymerization using functional initiator in the initiation step and/or functional nucleophile during the termination step.[136] This is

not usually the preferred synthetic route, particularly to couple biological molecules, because these are frequently sensitive to environmental conditions and can be degraded easily during the hydrolysis step, therefore losing activity. Hence, the post-functionalization of PEI chains is commonly employed despite being more tedious. The hydrolysis of POx creates secondary amines that can be used to graft residues able to improve biocompatibility, facilitate targeting and enhance buffering capacity, for instance.[137] The small molecules can be grafted at the polymer units and/or at the chain ends.[138–140] The amine groups allow a fairly large portfolio of post-functionalization reactions, thus permitting the manufacturing of a variety of derivatives starting either from the linear or branched configuration.

### ***6.3.2. Hydrophobization of PEI Chains***

The manufacturing of PEI chains with attached hydrophobic units can improve the delivery performance of transfecting agents since polycations with optimized degree of hydrophobicity are able to favorably interact with cell membranes containing lipids and cholesterol moieties, thereby leading to enhanced cellular uptake.[141] The hydrophobization of the chains are also capable to provide enhanced stability to the supramolecular assemblies.[141,142] The approach can also reduce the polymer-nucleic acid binding strength which is beneficial for intracellular nucleic acid unpacking and release of the genetic material.[143] The versatility of PEI chains enables the covalent link of various types of hydrophobic segments (alkyl chains, fatty acids and cholesteryl groups are some examples). The linking of cholesteryl chloroformate to primary amines was evidenced to increase PEI transfection efficiency,[144] although the substitution of the secondary amines was later suggested to be advantageous since primary amines would then remain available for DNA condensation.[131] The position

of the substituent has indeed a notable impact in the transfection performance of hydrophobically-modified PEI chains.[145] Overall, polyplexes produced from cholesterol-modified PEI chains demonstrates augmented cellular uptake and transfection efficiency. The fluorination of PEI chains was demonstrated to be an efficient strategy to provide enhanced serum stability and cellular uptake, although the degree of substitution has to be precisely controlled to balance cytotoxicity and intracellular delivery issues.[146] In the same framework, the modification of PEI with cholesterol (Chol) along with superhydrophobic perfluorinated (F) moieties (F-PEI-Chol) evidenced to provide enhanced stability, cellular uptake and siRNA silencing, as well as lower cytotoxicity compared to PEI, F-PEI and PEI-Chol.[147] The hydrophobic modification of low molar mass PEI by using short propionic acid (PrA) has been also hypothesized to enhance transfection efficiency. Optimized cellular uptake and efficient siRNA-induced silencing has been achieved with moderate degrees of substitution, highlighting that the balance between benefits and drawback is tiny, and remarkable increase in surface hydrophobicity might cause deleterious effects.[148] Likewise, higher transfection efficiency has been obtained by modifying PEI chains with shorter hydrophobic groups compared to longer ones, and relevant influence of the conjugation degree has been also underlined. [149] Beneficial effects have been also demonstrated *via* the substitution of low molar mass PEI with linoleic acid (LA) and  $\alpha$ -linolenic acid ( $\alpha$ LA) towards siRNA delivery to myeloid leukemia cells.[150] The grafting of oleic and stearic acids to branched PEI was evidenced to conduct to better siRNA binding and protection in serum-containing media, which accordingly assisted the siRNA delivery process.[151] The modification of PEI chains by using different types of fatty acids led to efficient targeting of pulmonary microvascular endothelium as mainly driven by surface charge features.[152] Overall, the hydrophobization of PEI

chains is generally reported to be a clever strategy to enhance the efficacy of PEI in nucleic acid delivery, as attributed at least to some extent to enhanced cellular uptake of the polyplexes.[153] The approach is also useful to assist intracellular steps of the gene delivery pathway, to reduce the cytotoxicity and to enhance the serum stability of resulting polyplexes. Nevertheless, it is almost unanimity that the degree of hydrophobicity is highly relevant and must be precisely tuned to balance benefits and drawbacks.

### ***6.3.3. Conjugation of Amino Acids and Peptides to PEI Chains***

Taking into account the conjugation of amino acids to PEI chains, the linear configuration of polyethylenimine (*l*PEI) was substituted with histidine *via* the Michael reaction, for instance. The histidinylated *l*PEI (His-*l*PEI) polyplexes allowed for efficient gene transfection as attributed to increased buffering capacity thanks to the presence of the imidazole function at the histidine moieties. The insertion of carboxylic functions also introduces negative charges to the polymeric gene delivery vector leading to lower cytotoxicity.[154] Moreover, cryo-TEM analysis of His-*l*PEI polyplexes revealed a less organized system compared to *l*PEI counterparts. The amorphous structure along with enhanced buffering capacity provided by the histidine residues possibly favor the intracellular dissociation of the assemblies and better cytosolic availability of pDNA, thus conducting to higher transfection efficiency.[155] Along these lines, it has been demonstrated recently that a mixture of *l*PEI and His-*l*PEI increases the transfecting activity in NIH3T3 fibroblasts and skeletal muscle cells, although at the current stage, the main driving force contributing to the phenomenon remains unclear.[156] The use of imidazole-containing units to improve transfection efficiency, particularly in serum environment, and reduce the cytotoxicity of PEI-based

polyplexes was also demonstrated with other agents (apart of amino acids) such as the urocanic acid.[157] The conjugation of other amino acids or their analogs similarly demonstrated enhanced transfection efficiency, particularly the grafting of glycolic acid to PEI chains, although the degree of substitution seems always to be a critical parameter.[158]

The peptidization of PEI chains was hypothesized to reduce cytotoxicity and enhance the cellular uptake of non-viral gene delivery vectors. The approach can be additionally used to improve the intracellular performance of gene carriers, such as by modifying PEI chains with pH triggered peptides. In this framework, the PEI functionalization with the C6M3 peptide enabled membrane lysis and efficient endosome release of the supramolecular assemblies, as well as high gene transfer capability along with biocompatibility to red blood cells at neutral pH.[159] The enhanced cellular uptake of peptide-modified PEI chains was also demonstrated for PEI decorated with Arg-Gly-Asp (RGD) which can target integrin receptors overexpressed in cancer cells.[160,161] The coating of PEI-DNA polyplexes with RGD or HIV-1 TAT peptides increased the cellular uptake of the therapeutic carriers and the transfection efficiency in neuronal cells. The experimental data additionally suggested augmented endosomal escape, pointing out that the modifications assist different steps of the delivery pathway.[162] The targeting capability has been similarly observed for PEI conjugate to the RPM peptide that targets preferably invasive colorectal cancer.[163]

#### ***6.3.4. Sugar Decoration of PEI Chains***

The engineering of bioactive glyconanostructures whose outer shell is decorated with sugar residues potentially enhances the cellular uptake of polyplexes *via* cell-surface reception, which can therefore augment the bioavailability of nucleic acids. The



cell-specific targeting can be used with carbohydrates due to their specificity to lectins that can be present in the cell surfaces.[164] Along these lines, enhanced cellular uptake of bioactive molecules was evidenced using oligosaccharide-shelled hyperbranched PEI complexed to ATP molecules,[165] and the strategy was considered to enhance the biocompatibility and *in vivo* gene delivery efficacy of polyplexes, for instance, by grafting maltose or maltotriose moieties.[166] Moreover, the sugar-decoration may provide shielding to the polyplexes making them less susceptible to opsonization, and offering more robust serum stability as required for systemic delivery systems.[167] The PEI chains were also galactosylated by reductive amination to target hepatocytes.[168] The sugar provided a shielding effect to the supramolecular aggregates, and the cytotoxicity was reduced by increasing the degree of galactosylation however, the transfection efficiency was evidenced to be highly affected by the degree of substitution, which indeed seems to be critical, regardless of the chemical nature of the substituent. The use of glycosylated PEI was demonstrated to provide enhanced gene transfer, although the efficacy is reduced in differentiated cells compared to undifferentiated counterparts.[169] The behavior was suggested to be related to impaired intracellular trafficking of the distinct polyplexes. The substitution of primary amine groups in bPEI chains with lactose residues was demonstrated by us to lead to a substantial reduction in cytotoxicity with a balanced effect in gene expression.[170] The simultaneous alkylcarboxylation and galactose conjugation to 25 kDa PEI is effective in the delivering of DNA to hepatocyte cells. The alkylcarboxylated chains presumably improve the hydrophilic-hydrophobic balance in the polycations which are uptaken in high yields due to the abundance of asialoglycoprotein receptors (ASGPRs) in liver parenchymal cells.[171] Enhanced uptake of galactosylated PEI by cells over-expressing ASGPRs was also evidenced for other derivatives.[172] PEI chains were

likewise functionalized with  $\beta$ -glucan and the derivative demonstrated enhanced cellular uptake and higher transfection efficiency compared to *b*PEI in RAW264.7 cells. The evidences suggest that the derivatives are able to recognize and bind to membrane receptors of such particular cell type.[173] The mannosylation of PEI chains was demonstrated to be useful for targeting specific cell types for self-amplifying RNA (saRNA) delivery and expression.[174] Overall, sugar decoration of PEI is able to enhance the cellular uptake of polyplexes due to the suitable affinity of the moieties to components present in the cell membranes, and the approach typically enhances serum stability and biocompatibility (increased cell viability is normally monitored).

#### ***6.3.5. Environmentally-Responsive PEI Chains***

Polyethylenimine is known to be an efficient gene delivery vector however, it is accepted that chemical modifications are useful to augment transfection rates and decrease reactions of the immune system. The strategy of using PEI derivatives with environmentally-responsive properties usually targets non-viral gene delivery vectors with high transfection efficiency, but lower cytotoxicity. The method can be used to shield the positively charged surface of PEI during systemic circulation, thereby avoiding the non-specific adsorption of negatively charged proteins (abundant in the bloodstream) which typically induces immune responses. Nevertheless, residual positive charges at the surface of polyplexes are desired to enhance cellular uptake *via* favorable electrostatic interactions. Thus, charge recovery can be reached at the interface with targeting cells by using environmentally-responsive PEI chains that respond to slightly acid microenvironments commonly found in tumor sites, for example. In this framework, shielded PEI-based polyplexes were constructed using aldehyde-containing PEG chains able to react with the amino groups forming stable Schiff bases in physiological environment. The shielding provided decreased

cytotoxicity, improved stability and prolonged circulation time. The PEG de-shielding was achieved by the cleavage of the chemical bonds at slightly acidic milieu, then allowing the recovery of positive charge with beneficial outputs with respect to cellular uptake and transfection activity.[175] The shielding of positively charged PEI/pDNA polyplexes was also reached by using zwitterionic polymers with charge conversion features.[176] Concerning the intercellular compartment, approaches to targeted PEI chains of high molar mass that can be degraded in the intracellular environment may conduct to gene vectors of lower toxicity.[177] In this context, biodegradable PEI derivatives were constructed by using different functional groups such as disulfide bonds,[178–180] and ketal,[181] imine,[182–184] amide,[185] ester,[186,187] hydrazone[188] and carbamate[189] linkers. These investigations overall reported reduced cytotoxicity and enhanced transfection rates regardless of the functional group providing degradability. Disulfide bonds were for instance used to link short PEI chains to produce longer ones. The intracellular degradation was induced by the presence of glutathione (GSH), and the release of less toxic PEI fragments was identified.[178] The reductive degradation is, therefore, manifested to be an innovative strategy in the design of polymeric non-viral vectors with improved efficacy and reduced levels of cytotoxicity. Zhang *et al.* demonstrated the use of reactive oxygen species (ROS)-degradable polymeric gene delivery vectors in the same perspective. Stimuli-responsive cross-linked OEI-TK<sub>x</sub> were manufactured using oligoethylenimine (OEI) *via* thioketal (TK) linkages that are cleaved selectively in ROS-rich environments (another common feature of tumor sites). The vectors induce lower cytotoxicity and enhanced gene transfection efficiency compared to PEI/DNA polyplexes. The performance was attributed to the cleavage of thioketal linkages and OEI-TK<sub>x</sub>/DNA disassembly in the intracellular ambient as triggered by high ROS-concentration mediated by

hypoxia.[190] Truthfully, the use of environmentally-responsive and degradable chains is possibly one of the best strategies to balance benefits and drawbacks of polymeric gene delivery vectors.[191]

#### **6.3.6. Shielding of PEI Chains**

The use of shielding agents is intended to provide extracellular stability to polyplexes, thus reducing aggregation in highly complex media such as plasma and accordingly, increasing the blood circulation time. Indeed, bare PEI polyplexes are susceptible to protein adsorption whenever swimming in biological fluids. The PEGylation approach is overall used in nanomedicine to provide stealth properties and colloidal stability, thus reducing the recognition of nanocarriers by the immune system,[192] as well as their cytotoxicity depending on the final composition.[193,194] Despite the outstanding advantage, it is widely reported in the literature that such a strategy reduces the cellular uptake mainly due to the reduction in the zeta potential of the assemblies, then decreasing the strength of cell adhesion. The presence of PEG also interferes in the nuclei acid condensation process.[167] Ultimately, it inevitably reduces the desired high transfection levels of gene delivery systems.[195] This can be to some extent balanced by precisely selecting PEG molar mass and degree of conjugation.[196,197] The advantages of optimized PEGylation have been demonstrated *in vivo*. Increased circulation time and gene transfer without significant toxicity in tumor bearing mice were observed for PEI-based PEGylated assemblies in comparison with non-PEGylated counterparts.[198] Yet, the searching for PEG substitutes is essential because it is widely accepted that the PEGylation is not able to completely prevent protein adsorption,[199] and that PEG triggers the activation of the complement system.[200] Additionally, the therapeutic efficacy of PEG-based

nanomedicines is usually compromised by the presence of PEG antibodies (anti-PEG) produced by the immune system,[201] thus inducing the so-called accelerated blood clearance (ABC).[202] Hence, PEG alternatives are claimed and amongst them, one finds different biocompatible polymers such as poly (2-oxazoline)s, polyglycerols, polyvinylpyrrolidone, polyacrylamides and zwitterionic polymers, particularly those based on sulfobetaine.[203] The use of copolymers containing PEtOx and PEI is useful to control the charge density of the polymer chains *via* partial acid hydrolysis with significant biological consequences.[204,205] In the same lines, the use of block copolymers PEI-*b*-PEtOx as gene vectors provided enhanced cell viability without reducing transfection rates compared to PEI equivalents,[206] and *l*PEI-*comb*-PEtOx polymers with different molar masses and grafting densities have been also proposed as vectors for gene delivery.[207] In such a case, the transfection efficiency was evidenced to be dependent on the morphology of the manufactured assemblies. The grafting strategy has been explored by Gwak *et al.* who demonstrated enhanced stability of polyplexes in the presence of competing polyanions, and nuclease protection by using the cationic amphiphilic copolymer poly(lactide-*co*-glycolide)-graft-polyethylenimine (PgP) as transfecting agent,[208] and copolymers made by poly(5-methyl-5-allyloxycarbonyl-trimethylene carbonate) and PEI (PMAC-*g*-PEI) were evidenced to provide enhanced transfection efficiency and lower cytotoxicity to 293T cells compared to PEI.[209] The further introduction of 5,5-dimethyl-trimethylene carbonate (DTC) enabled the control over charge density and hydrophobicity, notably affecting endosomal escape and nucleic acid unpacking.[210] The control over charge density on PEI-based polyplexes was similarly achieved *via* the physical adsorption of anionic polyelectrolyte layers.[211]

Besides the use of polymeric materials to shield PEI-based gene carriers, the goal can be also targeted by using small molecules such as *via* the succinylation of the polycation.[212] Zwitterion-like derivatives were reported to reduce the aggregation in serum media and increase transfection rates. The enhanced activity was also attributed to reduced polymer-nucleic acid interaction strength therefore enabling a smoother unpacking of the genetic material in the intracellular environment. Nevertheless, a required balanced with regard to nucleic acid condensation was highlighted, which is tuned by the neutralization of primary and secondary amines and accordingly, once again dependent on the degree of substitution of the amine groups. The succinylation of PEI chains also conduct to remarkably lower polymer toxicity compared to unmodified chains.[213]

### ***6.3.7. PEI Chains with Specific Targeting Ligands***

One widely investigated avenue towards the development of optimized non-viral vectors is the derivatization of polycations by using ligands to target specific cells. The use of molecules that are recognized by receptors present at the cell surfaces may intensify cell binding thereby enhancing cellular uptake and further gene expression. The investigations concerning active targeting rely mostly on genetic approaches for cancer treatment. The strategy has been exploited for instance using transferrin (Tf), folate (FR), integrins and epidermal growth factor (EGF) receptors, which are known to be overexpressed at the surface of a variety of cancer cells. Xie *et al.* for instance, functionalized PEI chains with transferrin (Tf-PEI) to optimize the gene transfection in asthmatics (transferrin receptors are overexpressed on cells responsible for this disease).[139] *In vitro* studies demonstrated notably enhanced cellular uptake and gene knockdown mediated by Tf-PEI polyplexes in human primary Activated T cells

(ATCs), and *in vivo* biodistribution of the polyplexes on a murine asthmatic model confirmed that Tf-PEI polyplexes can efficiently and selectively deliver siRNA to ATCs. The folate ligand is also widely used for cell targeting purposes. For example, folic acid was coupled to 1.8 kDa bPEI *via* two successive N-acylation reactions. The conjugates with relatively low substitution amounts (especially PEI-FA<sub>0.65</sub>) conducted to the smallest polyplexes at weight ratio = 3 and nearly 100-fold higher transfection efficiency compared to unsubstituted chains. The performance was attributed to folate receptor-mediated cellular uptake, better pDNA binding ability and favorable pH buffering capacity.[214] The folic acid targeting function was also evidenced in PEI-based ternary complexes manufactured for gene delivery purposes.[215] Guo and Lee similarly investigated PEI vectors functionalized with folate (FA).[216] PEI-FA vectors transfected KB cells at the same level compared to unmodified complexes. The efficiency was improved by using the PEGylation strategy conferring increased availability of the ligand thus optimizing cell recognition. Different ligands were evaluated to target EGF-overexpressing cells including different peptides and antibodies.[217–219] Concerning particularly the overexpression of  $\alpha\beta3$  integrin receptors on cancer cells, one example of targeting approach was the conjugation of L-thyroxine to PEI chains for pDNA delivery. These receptors hold binding sites for L-thyroxine and two-fold higher transfection rate compared to unmodified PEI was evidenced in cell lines overexpressing integrin.[220]

#### **6.4. Insights on the Formation and Structural Features of PEI-based/DNA Polyplexes**

Although a lot of efforts have been dedicated to optimize PEI-based polyplexes *via* the chemical modification of the polymer chains, fewer investigations have been

performed with regard to the formation and structural features of the electrostatic complexes, and the majority of them have been performed only by using unmodified chains. Significant discoveries have been shared, although the link with transfection activity has not been robustly identified so far. The presence of free PEI chains in optimized formulations for transfection seems to be well accepted as suggested theoretically[221] and repeatedly shown experimentally. Clamme *et al.* reported that ~86% of the PEI chains are freely diffusing in solution and that PEI/DNA polyplexes are composed by an average of 3.5 plasmids (5.1 kbp) and 30 PEI 25 kDa chains at N/P = 6.[30] These numbers are variable and the presence of PEI/DNA polyplexes composed on average by 8 to 32 pDNA ( $M_w = 2.1 \times 10^6 \text{ g.mol}^{-1}$ ) and  $70 \pm 25$  PEI chains (13.4 kDa) was determined by other authors using a combination of sedimentation velocity analysis and scanning force microscopy.[222] Recently, it has been highlighted that the number of loaded DNA copies can be precisely tuned by kinetically controlling the PEI/DNA assembly process, underscoring that charge neutralization is not a rate-limiting step, and that the assembly time is chiefly governed by chain folding and compaction of polyelectrolyte units. The control over the mixing conditions using flash nanocomplexation enabled the tuning over pDNA (~ 6 kbp) payload from 1.3 to 21.8 copies per particle with average hydrodynamic size ranging from 35 to 130 nm. The assemblies with intermediate payloads (6 to 10 pDNA copies *per* particle) evidenced to be more effective in mediating gene expression.[223] Indeed, the delivered number of copies is particularly relevant concerning the applicability of polyplex formulations. For instance, the number of copies to be delivered in large mammals is expected to be in the range of  $10^{13}$  copies/kg[224] thus corresponding to roughly  $2 \times 10^{-11}$  mol/kg or 100  $\mu\text{g/kg}$  of 10 kb pDNA. These numbers have to be considered only as rough estimations since they depend on many variables, including the size of the plasmid. Nevertheless,



the use of non-viral vectors is a clever strategy also in this regard since it can carry more than one DNA copy, which is the loading capacity of a viral vector (one copy *per* capsid).

Investigations concerning the dynamic behavior of PEI/DNA polyplexes were also performed. They are certainly relevant to better understand the levels of transfection efficiency reported for different assemblies. Lisitsyna *et al.* suggested recently that at  $N/P = 2$  all phosphate groups of DNA chains are bound to the nitrogen atoms of PEI, therefore forming a PEI/DNA core.[225] Further PEI addition results in positively charged shells. They evidenced a dynamic behavior of the complexes, where chains in the core and in the shell can be exchanged (PEI chains present in the shell can replace the ones attached to DNA in the core, and *vice-versa*). The PEI polyplexes are presumably much more dynamic than PLL counterparts and this can, at least to some extent, explain the favorable transfection using PEI polyplexes. Along these lines, it has been demonstrated that DNA chains can be exchanged between previously manufactured PEI-based polyplexes depending on the polymer/DNA interaction strength. This behavior has been observed particularly for *l*PEI partially substituted with histidine residues, whereas no exchange was evidenced in PLL polyplexes. The results simultaneously revealed that PEI-based polyplexes contain several DNA copies, and that remarkably different assemblies can be produced depending on the chemical features of the polymeric vector. Possibly, highly organized and well-compacted systems may disfavor further nucleic acid unpacking, thereby leading to reduced transfection capability.[226] This assumption has been highlighted by us while investigating His-*l*PEI and *l*PEI polyplexes as previously remarked.[155] Still concerning the internal structure of such type of assemblies, the presence of large amounts of solvent has been determined inside PEI/DNA polyplexes. These authors

highlighted that, apart from the N/P ratio, the concentration of the polyelectrolytes is highly relevant and, even at a fixed N/P ratio, size and charge of the electrostatic complexes are increasing functions of their concentration.[227]

Taking into account the mechanism of complex formation, the PEI/DNA interaction has been proposed to be associated with both groove binding and electrostatic forces, the later acting at the external phosphate backbone, leading to DNA condensation. The binding was evidenced to be pH-dependent,[228] similarly demonstrated by Ketola *et al.*, who also evidenced complete DNA condensation at N/P ~ 2.[229] The mechanism of complex formation changes from independent binding at N/P < 0.6 and pH 7.4 to cooperative binding at higher N/P ratios. On the other hand, the complex formation is cooperative at all N/P ratios at pH 5.2, therefore suggesting that possibly, the manufacturing of the complexes at lower pH can be beneficial, although the transfection is performed at biological pH. This is linked to a higher amount of protonated amine groups as pH decreases.

Concerning the structural features of PEI/DNA polyplexes at different N/P ratios, Mengarelli *et al.* reported a sequence of weak and strong complexation followed by charge inversion and further dissociation of large aggregates as the polycation concentration increases. Smaller and negatively charged complexes at low polymer concentrations are produced first. They are then condensed at higher polycation concentration, thereby forming large anionic aggregates at N/P ~ 1. They are further dissolved as linked to charge inversion at N/P > 1.[230] This has been similarly observed by Perevyazko *et al.* who evidenced incomplete DNA condensation and formation of primary PEI/DNA complexes at N/P < 1, their merging at N/P ~ 2 leading to the formation of large aggregates (~ 1 µm), and finally their progressive dissolution as N/P increases. Stable electrostatic complexes with average size of  $170 \pm 65$  nm were

produced at  $N/P > 10$ .<sup>[222]</sup> The interaction of DNA with hydrophobically-modified PEI chains was also evidenced to occur in three steps. The biomacromolecule is initially partially compacted, then micrometric aggregates appear and further, compacted and positively charged assemblies with  $R_H$  ranging from 52-86 nm were observed.<sup>[231]</sup> Overall, a number of investigations were devoted to the dynamics, thermodynamics and structural features of PEI-based polyplexes, although there is still a missing link between the findings and the transfection activity. Similar investigations to be performed using different PEI derivatives would be highly valuable to identify relevant differences that can potentially be connected to different degrees of transfection efficiency.

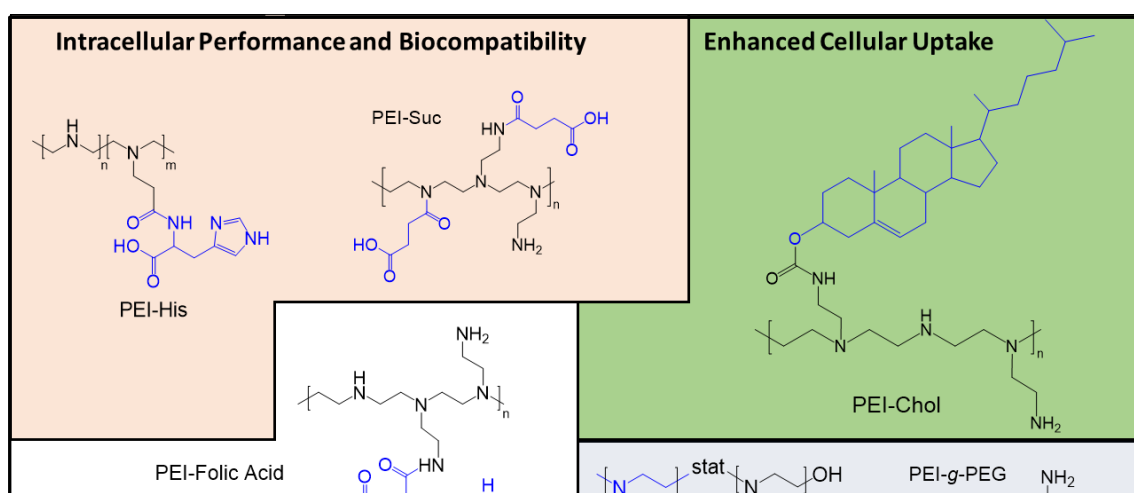
## **7. Current Status towards Marketed Products**

The origins of gene therapy remount to roughly 50 years ago with expectations and setbacks. At the present time, gene-based therapies are realistic to some extent, yet uncommon. This is at least partially justified by its focus, typically in rare disorders, then targeting a reduced number of patients. Such a specific feature raises remarkably the cost of treatments. The few number of commercially available products are essentially based on genetically modified viruses due to their natural ability to infect cells.<sup>[9]</sup> To the best of our knowledge, Onpattro (approved in the USA in August 2018) is the only formulation based on a non-viral vector with market authorization for *in vivo* human use.<sup>[232]</sup> This nanomedicine is based on lipid nanoparticles carrying RNA interference (RNAi) for the treatment of amyloid polyneuropathy. The majority of the gene therapies in clinical trials are indeed based on viral vectors (around 70%) and, within the class of non-viral vectors, they are produced mainly by using lipids. Although many of them were abandoned in the pathway from the clinical evaluation towards market authorization, various others are currently being tested. Concerning

polymer-based formulations, they are typically built by using a polycation (for nucleic acid condensation), PEG (for shielding purposes) and specific ligands (for cell targeting). This includes, for instance, CALAA-01 (first targeted polymer-based nanoparticle-carrying siRNA administered in humans) consisting of four components (siRNA, cyclodextrin, PEG and transferrin).[233,234]. PEI-based DNA vaccination against neuroblastoma and gene therapy for pancreatic ductal adenocarcinoma are currently listed in clinical trials (<https://www.clinicaltrials.gov>). PEI-based formulations in clinical evaluation can be also found with derivatives such as PEG-PEI-cholesterol.[235] Despite the clinical progresses, there is no polymer-based gene therapeutics with marketed authorization at the present time, as far as we know.

## CONCLUSIONS AND CRITICAL OPINION

The PEI-based non-viral vectors are efficient in the delivery of nucleic acids inside the cells, and potentially capable to substitute viral agents. The gene delivery by using non-viral vectors is nevertheless a multi-step process that poses many challenges, either regarding the extracellular or intracellular media. The advances in polymer and conjugate chemistry enabled the creation of a large number of PEI derivatives that were demonstrated to overwhelm some of the main difficulties. Figure 6 reports representative examples of approaches reported in the literature. Importantly, the derivatization is usually beneficial to different steps of the transfection process, although we highlight the main advantage.



**Figure 6.** Examples of PEI derivatives designed to overcome extracellular and intracellular barriers towards enhanced efficacy of PEI-based non-viral gene delivery systems.

Despite the advances and accomplishments, an ideal vector is hardly achieved since different steps in the delivery pathway commonly require conflicting properties (such as the extracellular stability and intracellular lability, the latter needed for nucleic acid unpacking). The complexity of systemic gene therapy therefore justifies the only moderate number of non-viral vectors that have progressed to clinical trials. The requirements of non-viral gene delivery vectors in the extracellular milieu are already decently understood however, those needed for efficient cytosolic delivery and nuclei internalization are still debatable. Further investigations based on the present level of knowledge may positively impact applications in the future. The chemical flexibility of PEI chains (either during the polymerization protocols or afterwards), advances in macromolecular chemistry and high-resolution imaging techniques will be useful to understand these fundamental aspects towards further optimizations. Additionally, more investigations concerning the dynamics, thermodynamics and structural parameters, particularly of PEI derivatives, would be valuable since these features can be further connected to the different levels of transfection efficiency hitherto evidenced. The gene

delivery by using polymeric platforms is undoubtedly a highly complex process and presumably, one will find the most promising vectors among those able to respond to the inherent features of the extracellular and intracellular environments to face the typically incompatible correlations.

## **CONFLICT OF INTEREST**

The authors declare no conflict of interest, financial or otherwise.

## **ACKNOWLEDGMENTS**

We acknowledge the sponsoring by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grant 2019/20470-8) and by the French National Funding Agency (ANR-17-CE18-0015-01-VINP). L.J.C.A and F.A.O acknowledge the fellowships granted by FAPESP (grants 2016/23844-8 and 2019/12944-0), and F.C.G acknowledges the fellowship granted by FAPESP (grant 2018/11038-2) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (grant 303268/2020-4).

## **REFERENCES**

- [1] L.W. Seymour, A.J. Thrasher, Gene Therapy Matures in the Clinic, *Nat. Biotechnol.* 30 (2012) 588–593. <https://doi.org/10.1038/nbt.2290>.
- [2] G. Shim, D. Kim, G.T. Park, H. Jin, S.-K. Suh, Y.-K. Oh, Therapeutic gene editing: delivery and regulatory perspectives, *Acta Pharmacol. Sin.* 38 (2017) 738–753. <https://doi.org/10.1038/aps.2017.2>.
- [3] H. Erfle, B. Neumann, U. Liebel, P. Rogers, M. Held, T. Walter, J. Ellenberg, R. Pepperkok, Reverse transfection on cell arrays for high content screening microscopy, *Nat. Protoc.* 2 (2007) 392–399. <https://doi.org/10.1038/nprot.2006.483>.

- [4] H. Lomas, J. Du, I. Canton, J. Madsen, N. Warren, S.P. Armes, A.L. Lewis, G. Battaglia, Efficient Encapsulation of Plasmid DNA in pH-Sensitive PMPC-PDPA Polymersomes: Study of the Effect of PDPA Block Length on Copolymer-DNA Binding Affinity, *Macromol. Biosci.* 10 (2010) 513–530. <https://doi.org/10.1002/mabi.201000083>.
- [5] B.E. Houk, R. Martin, G. Hochhaus, J.A. Hughes, Pharmacokinetics of Plasmid DNA in the Rat, *Pharm Res.* 18 (2001) 67–74. <https://doi.org/10.1023/a:1011078711008>.
- [6] H. Yin, R.L. Kanasty, A. a. Eltoukhy, A.J. Vegas, J.R. Dorkin, D.G. Anderson, Non-Viral Vectors for Gene-Based Therapy, *Nat. Rev. Genet.* 15 (2014) 541–555. <https://doi.org/10.1038/nrg3763>.
- [7] T. Hollon, Researchers and Regulators Reflect on First Gene Therapy Death, *Nat. Med.* 6 (2000) 6–6. <https://doi.org/10.1038/71545>.
- [8] S.H. Orkin, P. Reilly, Paying for Future Success in Gene Therapy, *Science.* 352 (2016) 1059–1061. <https://doi.org/10.1126/science.aaf4770>.
- [9] N. AL Qtaish, I. Gallego, I. Villate-Beitia, M. Sainz-Ramos, T.B. López-Méndez, S. Grijalvo, R. Eritja, C. Soto-Sánchez, G. Martínez-Navarrete, E. Fernández, G. Puras, J.L. Pedraz, Niosome-Based Approach for in Situ Gene Delivery to Retina and Brain Cortex as Immune-Privileged Tissues, *Pharmaceutics.* 12 (2020) 198. <https://doi.org/10.3390/pharmaceutics12030198>.
- [10] A. Rolland, Gene Medicines: The End of the Beginning?, *Adv. Drug Deliv. Rev.* 57 (2005) 669–673. <https://doi.org/10.1016/j.addr.2005.01.002>.
- [11] R. Waehler, S.J. Russell, D.T. Curiel, Engineering Targeted Viral Vectors for Gene Therapy, *Nat. Rev. Genet.* 8 (2007) 573–587. <https://doi.org/10.1038/nrg2141>.

- [12] A. Berns, Good News for Gene Therapy, *N. Engl. J. Med.* 350 (2004) 1679–1680. <https://doi.org/10.1056/NEJMcibr040341>.
- [13] R. Deng, Y. Yue, F. Jin, Y. Chen, H.-F. Kung, M.C.M. Lin, C. Wu, Revisit the Complexation of PEI and DNA - How to Make Low Cytotoxic and Highly Efficient PEI Gene Transfection Non-Viral Vectors with a Controllable Chain Length and Structure?, *J. Control. Release.* 140 (2009) 40–46. <https://doi.org/10.1016/j.jconrel.2009.07.009>.
- [14] J. Han, S.K. Kim, T.-S. Cho, J.-C. Lee, H.S. Joung, Polyplex Formation of Calf Thymus DNA with Branched and Linear Polyethyleneimine, *Macromol. Res.* 12 (2004) 501–506. <https://doi.org/10.1007/BF03218434>.
- [15] P. Zhang, E. Wagner, History of Polymeric Gene Delivery Systems, *Top. Curr. Chem.* 375 (2017) 1–39. <https://doi.org/10.1007/s41061-017-0112-0>.
- [16] B.D. Monnery, M. Wright, R. Cavill, R. Hoogenboom, S. Shaunak, J.H.G. Steinke, M. Thanou, Cytotoxicity of Polycations: Relationship of Molecular Weight and the Hydrolytic Theory of the Mechanism of Toxicity, *Int. J. Pharm.* 521 (2017) 249–258. <https://doi.org/10.1016/j.ijpharm.2017.02.048>.
- [17] D. Fischer, Y. Li, B. Ahlemeyer, J. Krieglstein, T. Kissel, In Vitro Cytotoxicity Testing of Polycations: Influence of Polymer Structure on Cell Viability and Hemolysis, *Biomaterials.* 24 (2003) 1121–1131. [https://doi.org/10.1016/S0142-9612\(02\)00445-3](https://doi.org/10.1016/S0142-9612(02)00445-3).
- [18] G. Grandinetti, A.E. Smith, T.M. Reineke, Membrane and Nuclear Permeabilization by Polymeric pDNA Vehicles: Efficient Method for Gene Delivery or Mechanism of Cytotoxicity?, *Mol. Pharm.* 9 (2012) 523–538. <https://doi.org/10.1021/mp200368p>.
- [19] K. Lundstrom, Viral Vectors in Gene Therapy, *Diseases.* 6 (2018) 42.



<https://doi.org/10.3390/diseases6020042>.

- [20] B. Shi, M. Zheng, W. Tao, R. Chung, D. Jin, D. Ghaffari, O.C. Farokhzad, Challenges in DNA Delivery and Recent Advances in Multifunctional Polymeric DNA Delivery Systems, *Biomacromolecules*. 18 (2017) 2231–2246. <https://doi.org/10.1021/acs.biomac.7b00803>.
- [21] O.M. Merkel, R. Urbanics, P. Bedőcs, Z. Rozsnyay, L. Rosivall, M. Toth, T. Kissel, J. Szebeni, In Vitro and In Vivo Complement Activation and Related Anaphylactic Effects Associated with Polyethylenimine and Polyethylenimine-Graft-Poly(ethylene glycol) Block Copolymers, *Biomaterials*. 32 (2011) 4936–4942. <https://doi.org/10.1016/j.biomaterials.2011.03.035>.
- [22] J. Rejman, V. Oberle, I.S. Zuhorn, D. Hoekstra, Size-Dependent Internalization of Particles via the Pathways of Clathrin- and Caveolae-mediated Endocytosis, *Biochem. J.* 377 (2004) 159–169. <https://doi.org/10.1042/bj20031253>.
- [23] Z. Wang, C. Tiruppathi, R.D. Minshall, A.B. Malik, Size and Dynamics of Caveolae Studied Using Nanoparticles in Living Endothelial Cells, *ACS Nano*. 3 (2009) 4110–4116. <https://doi.org/10.1021/nn9012274>.
- [24] I. Canton, G. Battaglia, Endocytosis at the nanoscale, *Chem. Soc. Rev.* 41 (2012) 2718. <https://doi.org/10.1039/c2cs15309b>.
- [25] L. Billiet, J.-P. Gomez, M. Berchel, P.-A. Jaffrès, T. Le Gall, T. Montier, E. Bertrand, H. Cheradame, P. Guégan, M. Mével, B. Pitard, T. Benvegny, P. Lehn, C. Pichon, P. Midoux, Gene Transfer by Chemical Vectors, and Endocytosis routes of Polyplexes, Lipoplexes and Lipopolyplexes in a Myoblast Cell Line, *Biomaterials*. 33 (2012) 2980–2990. <https://doi.org/10.1016/j.biomaterials.2011.12.027>.
- [26] J. Rejman, A. Bragonzi, M. Conese, Role of Clathrin- and Caveolae-Mediated

- Endocytosis in Gene Transfer Mediated by Lipo- and Polyplexes, *Mol. Ther.* 12 (2005) 468–474. <https://doi.org/10.1016/j.ymthe.2005.03.038>.
- [27] D. Vercauteren, M. Piest, L.J. van der Aa, M. Al Soraj, A.T. Jones, J.F.J. Engbersen, S.C. De Smedt, K. Braeckmans, Flotillin-Dependent Endocytosis and a Phagocytosis-Like Mechanism for Cellular Internalization of Disulfide-Based Poly(amido amine)/DNA Polyplexes, *Biomaterials*. 32 (2011) 3072–3084. <https://doi.org/10.1016/j.biomaterials.2010.12.045>.
- [28] M.N. Hossen, L. Wang, H.R. Chinthalapally, J.D. Robertson, K.-M. Fung, S. Wilhelm, M. Bieniasz, R. Bhattacharya, P. Mukherjee, Switching the Intracellular Pathway and Enhancing the Therapeutic Efficacy of Small Interfering RNA by Auroliposome, *Sci. Adv.* 6 (2020) eaba5379. <https://doi.org/10.1126/sciadv.aba5379>.
- [29] A.L. Kiss, E. Botos, Endocytosis via Caveolae: Alternative Pathway with Distinct Cellular Compartments to Avoid Lysosomal Degradation?, *J. Cell. Mol. Med.* 13 (2009) 1228–1237. <https://doi.org/10.1111/j.1582-4934.2009.00754.x>.
- [30] J.P. Clamme, J. Azoulay, Y. Mély, Monitoring of the Formation and Dissociation of Polyethylenimine/DNA Complexes by Two Photon Fluorescence Correlation Spectroscopy, *Biophys. J.* 84 (2003) 1960–1968. [https://doi.org/10.1016/S0006-3495\(03\)75004-8](https://doi.org/10.1016/S0006-3495(03)75004-8).
- [31] C.E. Gallops, C. Yu, J.D. Ziebarth, Y. Wang, Effect of the Protonation Level and Ionic Strength on the Structure of Linear Polyethyleneimine, *ACS Omega*. 4 (2019) 7255–7264. <https://doi.org/10.1021/acsomega.9b00066>.
- [32] H. Yin, K.J. Kauffman, D.G. Anderson, Delivery Technologies for Genome Editing, *Nat. Rev. Drug Discov.* 16 (2017) 387–399. <https://doi.org/10.1038/nrd.2016.280>.

- [33] H. Andersen, L. Parhamifar, A.C. Hunter, V. Shahin, S.M. Moghimi, AFM Visualization of Sub-50 nm Polyplex Disposition to the Nuclear Pore Complex Without Compromising the Integrity of the Nuclear Envelope, *J. Control. Release.* 244 (2016) 24–29. <https://doi.org/10.1016/j.jconrel.2016.11.008>.
- [34] S. Liang, X.-Z. Yang, X.-J. Du, H.-X. Wang, H.-J. Li, W.-W. Liu, Y.-D. Yao, Y.-H. Zhu, Y.-C. Ma, J. Wang, E.-W. Song, Optimizing the Size of Micellar Nanoparticles for Efficient siRNA Delivery, *Adv. Funct. Mater.* 25 (2015) 4778–4787. <https://doi.org/10.1002/adfm.201501548>.
- [35] T. Bus, A. Traeger, U.S. Schubert, The Great Escape: How Cationic Polyplexes Overcome the Endosomal Barrier, *J. Mater. Chem. B.* 6 (2018) 6904–6918. <https://doi.org/10.1039/C8TB00967H>.
- [36] Y. Jiang, Q. Lu, Y. Wang, E. Xu, A. Ho, P. Singh, Y. Wang, Z. Jiang, F. Yang, G.T. Tietjen, P. Cresswell, W.M. Saltzman, Quantitating Endosomal Escape of a Library of Polymers for mRNA Delivery, *Nano Lett.* 20 (2020) 1117–1123. <https://doi.org/10.1021/acs.nanolett.9b04426>.
- [37] A.M. Funhoff, C.F. van Nostrum, G. a. Koning, N.M.E. Schuurmans-Nieuwenbroek, D.J. a Crommelin, W.E. Hennink, Endosomal Escape of Polymeric Gene Delivery Complexes Is Not Always Enhanced by Polymers Buffering at Low pH, *Biomacromolecules.* 5 (2004) 32–39. <https://doi.org/10.1021/bm034041+>.
- [38] R. V Benjaminsen, M. a Matthebjerg, J.R. Henriksen, S.M. Moghimi, T.L. Andresen, The Possible “Proton Sponge ” Effect of Polyethylenimine (PEI) Does Not Include Change in Lysosomal pH, *Mol. Ther.* 21 (2013) 149–157. <https://doi.org/10.1038/mt.2012.185>.
- [39] Y.-Y. Won, R. Sharma, S.F. Konieczny, Missing Pieces in Understanding the

- Intracellular Trafficking of Polycation/DNA Complexes, *J. Control. Release.* 139 (2009) 88–93. <https://doi.org/10.1016/j.jconrel.2009.06.031>.
- [40] Z.U. Rehman, D. Hoekstra, I.S. Zuhorn, Mechanism of Polyplex- and Lipoplex-Mediated Delivery of Nucleic Acids: Real-Time Visualization of Transient Membrane Destabilization without Endosomal Lysis, *ACS Nano.* 7 (2013) 3767–3777. <https://doi.org/10.1021/nn3049494>.
- [41] L.J.C. Albuquerque, C.E. de Castro, K.A. Riske, M.C.C. da Silva, P.I.R. Muraro, V. Schmidt, C. Giacomelli, F.C. Giacomelli, Gene Transfection Mediated by Cationomers Requires Free Highly Charged Polymer Chains To Overcome Intracellular Barriers, *Biomacromolecules.* 18 (2017) 1918–1927. <https://doi.org/10.1021/acs.biomac.7b00344>.
- [42] S. Vaidyanathan, J. Chen, B.G. Orr, M.M. Banaszak Holl, Cationic Polymer Intercalation into the Lipid Membrane Enables Intact Polyplex DNA Escape from Endosomes for Gene Delivery, *Mol. Pharm.* 13 (2016) 1967–1978. <https://doi.org/10.1021/acs.molpharmaceut.6b00139>.
- [43] D.K. Bonner, X. Zhao, H. Buss, R. Langer, P.T. Hammond, Crosslinked Linear Polyethylenimine Enhances Delivery of DNA to the Cytoplasm, *J. Control. Release.* 167 (2013) 101–107. <https://doi.org/10.1016/j.jconrel.2012.09.004>.
- [44] Y. Yue, C. Wu, Progress and Perspectives in Developing Polymeric Vectors for In Vitro Gene Delivery, *Biomater. Sci.* 1 (2013) 152–170. <https://doi.org/10.1039/C2BM00030J>.
- [45] L.M.P. Vermeulen, S.C. De Smedt, K. Remaut, K. Braeckmans, The Proton Sponge Hypothesis: Fable or Fact?, *Eur. J. Pharm. Biopharm.* 129 (2018) 184–190. <https://doi.org/10.1016/j.ejpb.2018.05.034>.
- [46] R. Fraley, S. Subramani, P. Berg, D. Papahadjopoulos, Introduction of

- Liposome-Encapsulated SV40 DNA into Cells., *J. Biol. Chem.* 255 (1980) 10431–10435. [https://doi.org/10.1016/S0021-9258\(19\)70482-7](https://doi.org/10.1016/S0021-9258(19)70482-7).
- [47] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielsen, Lipofection: a Highly Efficient, Lipid-Mediated DNA-Transfection Procedure, *Proc. Natl. Acad. Sci.* 84 (1987) 7413–7417. <https://doi.org/10.1073/pnas.84.21.7413>.
- [48] R. Leventis, J.R. Silvius, Interactions of Mammalian Cells with Lipid Dispersions Containing Novel Metabolizable Cationic Amphiphiles, *Biochim. Biophys. Acta - Biomembr.* 1023 (1990) 124–132. [https://doi.org/10.1016/0005-2736\(90\)90017-I](https://doi.org/10.1016/0005-2736(90)90017-I).
- [49] H. San, Z. Yang, V.J. Pompili, M.L. Jaffe, G.E. Plautz, L. Xu, J.H. Felgner, C.J. Wheeler, P.L. Felgner, X. Gao, L. Huang, D. Gordon, G.J. Nabel, E.G. Nabel, Safety and Short-Term Toxicity of a Novel Cationic Lipid Formulation for Human Gene Therapy, *Hum. Gene Ther.* 4 (1993) 781–788. <https://doi.org/10.1089/hum.1993.4.6-781>.
- [50] H. Farhood, N. Serbina, L. Huang, The Role of dioleoyl Phosphatidylethanolamine in Cationic Liposome Mediated Gene Transfer, *Biochim. Biophys. Acta - Biomembr.* 1235 (1995) 289–295. [https://doi.org/10.1016/0005-2736\(95\)80016-9](https://doi.org/10.1016/0005-2736(95)80016-9).
- [51] Z. Du, M.M. Munye, A.D. Tagalakis, M.D.I. Manunta, S.L. Hart, The Role of the Helper Lipid on the DNA Transfection Efficiency of Lipopolyplex Formulations, *Sci. Rep.* 4 (2015) 7107. <https://doi.org/10.1038/srep07107>.
- [52] S.C. Semple, S.K. Klimuk, T.O. Harasym, M.J. Hope, Lipid-Based Formulations of Antisense Oligonucleotides for Systemic Delivery Applications, in: 2000: pp. 322–341. [https://doi.org/10.1016/S0076-6879\(00\)13020-4](https://doi.org/10.1016/S0076-6879(00)13020-4).

- [53] J.P. Behr, B. Demeneix, J.P. Loeffler, J. Perez-Mutul, Efficient Gene Transfer Into Mammalian Primary Endocrine Cells with Lipopolyamine-Coated DNA., *Proc. Natl. Acad. Sci.* 86 (1989) 6982–6986. <https://doi.org/10.1073/pnas.86.18.6982>.
- [54] D.A. Balazs, W. Godbey, Liposomes for Use in Gene Delivery, *J. Drug Deliv.* 2011 (2011) 1–12. <https://doi.org/10.1155/2011/326497>.
- [55] X. Gao, L. Huang, A Novel Cationic Liposome Reagent for Efficient Transfection of Mammalian Cells, *Biochem. Biophys. Res. Commun.* 179 (1991) 280–285. [https://doi.org/10.1016/0006-291X\(91\)91366-K](https://doi.org/10.1016/0006-291X(91)91366-K).
- [56] L. Ciani, S. Ristori, A. Salvati, L. Calamai, G. Martini, DOTAP/DOPE and DC-Chol/DOPE Lipoplexes for Gene Delivery: Zeta Potential Measurements and Electron Spin Resonance Spectra, *Biochim. Biophys. Acta - Biomembr.* 1664 (2004) 70–79. <https://doi.org/10.1016/j.bbamem.2004.04.003>.
- [57] J. Buck, P. Grossen, P.R. Cullis, J. Huwyler, D. Witzigmann, Lipid-Based DNA Therapeutics: Hallmarks of Non-Viral Gene Delivery, *ACS Nano.* 13 (2019) 3754–3782. <https://doi.org/10.1021/acsnano.8b07858>.
- [58] M.T. Luiz, L.B. Tofani, V.H.S. Araújo, L.D. Di Filippo, J.L. Duarte, J.M. Marchetti, M. Chorilli, Gene Therapy Based on Lipid Nanoparticles as Non-Viral Vectors for Gliomas Treatment, *Curr. Gene Ther.* 20 (2021). <https://doi.org/10.2174/1566523220999201230205126>.
- [59] Y. Xu, F.C. Szoka, Mechanism of DNA Release from Cationic Liposome/DNA Complexes Used in Cell Transfection, *Biochemistry.* 35 (1996) 5616–5623. <https://doi.org/10.1021/bi9602019>.
- [60] C. Zylberberg, K. Gaskill, S. Pasley, S. Matosevic, Engineering Liposomal Nanoparticles for Targeted Gene Therapy, *Gene Ther.* 24 (2017) 441–452.

- <https://doi.org/10.1038/gt.2017.41>.
- [61] R.B. Merrifield, Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide, *J. Am. Chem. Soc.* 85 (1963) 2149–2154. <https://doi.org/10.1021/ja00897a025>.
- [62] R.E. Taylor, M. Zahid, Cell Penetrating Peptides, Novel Vectors for Gene Therapy, *Pharmaceutics*. 12 (2020) 1–21. <https://doi.org/10.3390/pharmaceutics12030225>.
- [63] M.M. Khan, N. Filipczak, V.P. Torchilin, Cell Penetrating Peptides: A Versatile Vector For Co-Delivery of Drug and Genes in Cancer, *J. Control. Release*. (2020). <https://doi.org/10.1016/j.jconrel.2020.11.028>.
- [64] Ü. Langel, CPP, Cell-Penetrating Peptides, Springer Singapore, Singapore, 2019. <https://doi.org/10.1007/978-981-13-8747-0>.
- [65] H. Derakhshankhah, S. Jafari, Cell Penetrating Peptides: A Concise Review with Emphasis on Biomedical Applications, *Biomed. Pharmacother.* 108 (2018) 1090–1096. <https://doi.org/10.1016/j.biopha.2018.09.097>.
- [66] S. Sabouri-Rad, R.K. Oskuee, A. Mahmoodi, L. Gholami, B. Malaekheh-Nikouei, The Effect of Cell Penetrating Peptides on Transfection Activity and Cytotoxicity of Polyallylamine, *BioImpacts*. 7 (2017) 139–145. <https://doi.org/10.15171/bi.2017.17>.
- [67] D. Derossi, A.H. Joliot, G. Chassaing, A. Prochiantz, The Third Helix of the Antennapedia Homeodomain Translocates Through Biological Membranes, *J. Biol. Chem.* 269 (1994) 10444–10450.
- [68] B. Christiaens, P. Dubruel, J. Grooten, M. Goethals, J. Vandekerckhove, E. Schacht, M. Rosseneu, Enhancement of Polymethacrylate-Mediated Gene Delivery by Penetratin, *Eur. J. Pharm. Sci.* 24 (2005) 525–537.

- <https://doi.org/10.1016/j.ejps.2005.01.004>.
- [69] D. Schaffert, N. Badgular, E. Wagner, Novel Fmoc-Polyamino Acids for Solid-Phase Synthesis of Defined Polyamidoamines, *Org. Lett.* 13 (2011) 1586–1589. <https://doi.org/10.1021/ol200381z>.
- [70] P. Zhang, B. Steinborn, U. Lächelt, S. Zahler, E. Wagner, Lipo-Oligomer Nanoformulations for Targeted Intracellular Protein Delivery, *Biomacromolecules.* 18 (2017) 2509–2520. <https://doi.org/10.1021/acs.biomac.7b00666>.
- [71] I. Truebenbach, W. Zhang, Y. Wang, S. Kern, M. Höhn, S. Reinhard, J. Gorges, U. Kazmaier, E. Wagner, Co-delivery of Pretubulysin and siEG5 to EGFR Overexpressing Carcinoma Cells, *Int. J. Pharm.* 569 (2019) 118570. <https://doi.org/10.1016/j.ijpharm.2019.118570>.
- [72] C. Troiber, D. Edinger, P. Kos, L. Schreiner, R. Kläger, A. Herrmann, E. Wagner, Stabilizing Effect of Tyrosine Trimers on pDNA and siRNA Polyplexes, *Biomaterials.* 34 (2013) 1624–1633. <https://doi.org/10.1016/j.biomaterials.2012.11.021>.
- [73] X. Liu, P. Zhang, D. He, W. Rödl, T. Preiß, J.O. Rädler, E. Wagner, U. Lächelt, pH-Reversible Cationic RNase A Conjugates for Enhanced Cellular Delivery and Tumor Cell Killing, *Biomacromolecules.* 17 (2016) 173–182. <https://doi.org/10.1021/acs.biomac.5b01289>.
- [74] J. Luo, E. Wagner, Y. Wang, Artificial Peptides for Antitumoral siRNA Delivery, *J. Mater. Chem. B.* 8 (2020) 2020–2031. <https://doi.org/10.1039/C9TB02756D>.
- [75] Y. Wang, J. Luo, I. Truebenbach, S. Reinhard, P.M. Klein, M. Höhn, S. Kern, S. Morys, D.M. Loy, E. Wagner, W. Zhang, Double Click-Functionalized siRNA



- Polyplexes for Gene Silencing in Epidermal Growth Factor Receptor-Positive Tumor Cells, *ACS Biomater. Sci. Eng.* 6 (2020) 1074–1089. <https://doi.org/10.1021/acsbomaterials.9b01904>.
- [76] D. Schaffert, C. Troiber, E.E. Salcher, T. Fröhlich, I. Martin, N. Badgajar, C. Dohmen, D. Edinger, R. Kläger, G. Maiwald, K. Farkasova, S. Seeber, K. Jahn-Hofmann, P. Hadwiger, E. Wagner, Solid-Phase Synthesis of Sequence-Defined T-, i-, and U-Shape Polymers for pDNA and siRNA Delivery, *Angew. Chemie Int. Ed.* 50 (2011) 8986–8989. <https://doi.org/10.1002/anie.201102165>.
- [77] X.-L. Wang, S. Ramusovic, T. Nguyen, Z.-R. Lu, Novel Polymerizable Surfactants with pH-Sensitive Amphiphilicity and Cell Membrane Disruption for Efficient siRNA Delivery, *Bioconjug. Chem.* 18 (2007) 2169–2177. <https://doi.org/10.1021/bc700285q>.
- [78] A.S. Malamas, M. Gujrati, C.M. Kummitha, R. Xu, Z.-R. Lu, Design and Evaluation of New pH-Sensitive Amphiphilic Cationic Lipids for siRNA Delivery, *J. Control. Release.* 171 (2013) 296–307. <https://doi.org/10.1016/j.jconrel.2013.06.019>.
- [79] J. Haensler, F.C. Szoka, Polyamidoamine Cascade Polymers Mediate Efficient Transfection of Cells in Culture, *Bioconjug. Chem.* 4 (1993) 372–379. <https://doi.org/10.1021/bc00023a012>.
- [80] M. Ohsaki, T. Okuda, A. Wada, T. Hirayama, T. Niidome, H. Aoyagi, In Vitro Gene Transfection Using Dendritic Poly(L-lysine), *Bioconjug. Chem.* 13 (2002) 510–517. <https://doi.org/10.1021/bc015525a>.
- [81] M. Studzian, A. Szulc, A. Janaszewska, D. Appelhans, Ł. Pułaski, B. Klajnert-Maculewicz, Mechanisms of Internalization of Maltose-Modified Poly(propyleneimine) Glycodendrimers into Leukemic Cell Lines,

- Biomacromolecules. 18 (2017) 1509–1520.  
<https://doi.org/10.1021/acs.biomac.7b00046>.
- [82] C. Dufès, I.F. Uchegbu, A.G. Schatzlein, Dendrimers in Gene Delivery, *Adv. Drug Deliv. Rev.* 57 (2005) 2177–2202.  
<https://doi.org/10.1016/j.addr.2005.09.017>.
- [83] B.H. Zinselmeyer, S.P. Mackay, A.G. Schatzlein, I.F. Uchegbu, The Lower-Generation Polypropylenimine Dendrimers Are Effective Gene-Transfer Agents, *Pharm. Res.* 19 (2002) 960–967. <https://doi.org/10.1023/a:1016458104359>.
- [84] M. Yamagata, T. Kawano, K. Shiba, T. Mori, Y. Katayama, T. Niidome, Structural Advantage of Dendritic Poly(l-lysine) for Gene Delivery into Cells, *Bioorg. Med. Chem.* 15 (2007) 526–532.  
<https://doi.org/10.1016/j.bmc.2006.09.033>.
- [85] N. Weber, P. Ortega, M.I. Clemente, D. Shcharbin, M. Bryszewska, F.J. de la Mata, R. Gómez, M.A. Muñoz-Fernández, Characterization of Carbosilane Dendrimers as Effective Carriers of siRNA to HIV-Infected Lymphocytes, *J. Control. Release.* 132 (2008) 55–64.  
<https://doi.org/10.1016/j.jconrel.2008.07.035>.
- [86] M.J. Cloninger, Biological Applications of Dendrimers, *Curr. Opin. Chem. Biol.* 6 (2002) 742–748. [https://doi.org/10.1016/S1367-5931\(02\)00400-3](https://doi.org/10.1016/S1367-5931(02)00400-3).
- [87] H. Arima, K. Motoyama, T. Higashi, Sugar-Appended Polyamidoamine Dendrimer Conjugates with Cyclodextrins as Cell-Specific Non-Viral Vectors, *Adv. Drug Deliv. Rev.* 65 (2013) 1204–1214.  
<https://doi.org/10.1016/j.addr.2013.04.001>.
- [88] J. Chen, X. Guan, Y. Hu, H. Tian, X. Chen, Peptide-Based and Polypeptide-Based Gene Delivery Systems, *Top. Curr. Chem.* 375 (2017) 32.

<https://doi.org/10.1007/s41061-017-0115-x>.

- [89] D. Voet, J.G. Voet, *Biochemistry*, 2<sup>o</sup>, John Wiley and Sons, New York, 1995.
- [90] P. Midoux, M. Monsigny, Efficient Gene Transfer by Histidylated Polylysine/pDNA Complexes, *Bioconjug. Chem.* 10 (1999) 406–411. <https://doi.org/10.1021/bc9801070>.
- [91] I. Conejos-Sánchez, E. Gallon, A. Niño-Pariente, J.A. Smith, A.G. De la Fuente, L. Di Canio, S. Pluchino, R.J.M. Franklin, M.J. Vicent, Polyornithine-Based Polyplexes to Boost Effective Gene Silencing in CNS Disorders, *Nanoscale*. 12 (2020) 6285–6299. <https://doi.org/10.1039/C9NR06187H>.
- [92] Y. Zhou, S. Han, Z. Liang, M. Zhao, G. Liu, J. Wu, Progress in Arginine-Based Gene Delivery Systems, *J. Mater. Chem. B.* 8 (2020) 5564–5577. <https://doi.org/10.1039/D0TB00498G>.
- [93] E. Ramsay, M. Gumbleton, Polylysine and Polyornithine Gene Transfer Complexes: A Study of Complex Stability and Cellular Uptake as a Basis for their Differential in-vitro Transfection Efficiency, *J. Drug Target.* 10 (2002) 1–9. <https://doi.org/10.1080/10611860290007487>.
- [94] R.A. Kelley, S.M. Conley, R. Makkia, J.N. Watson, Z. Han, M.J. Cooper, M.I. Naash, DNA Nanoparticles Are Safe and Nontoxic in Non-Human Primate Eyes, *Int. J. Nanomedicine*. Volume 13 (2018) 1361–1379. <https://doi.org/10.2147/IJN.S157000>.
- [95] Z. Han, S.M. Conley, R. Makkia, J. Guo, M.J. Cooper, M.I. Naash, Comparative Analysis of DNA Nanoparticles and AAVs for Ocular Gene Delivery, *PLoS One*. 7 (2012) e52189. <https://doi.org/10.1371/journal.pone.0052189>.
- [96] N.J. Boylan, J.S. Suk, S.K. Lai, R. Jelinek, M.P. Boyle, M.J. Cooper, J. Hanes, Highly Compacted DNA Nanoparticles with Low MW PEG Coatings: In Vitro,

- Ex Vivo and In Vivo Evaluation, *J. Control. Release.* 157 (2012) 72–79.  
<https://doi.org/10.1016/j.jconrel.2011.08.031>.
- [97] J.M. Layman, S.M. Ramirez, M.D. Green, T.E. Long, Influence of Polycation Molecular Weight on Poly(2-dimethylaminoethyl methacrylate)-Mediated DNA Delivery In Vitro, *Biomacromolecules.* 10 (2009) 1244–1252.  
<https://doi.org/10.1021/bm9000124>.
- [98] A.M. Funhoff, C.F. van Nostrum, M.C. Lok, J.A.W. Kruijtzter, D.J.A. Crommelin, W.E. Hennink, Cationic Polymethacrylates with Covalently Linked Membrane Destabilizing Peptides as Gene Delivery Vectors, *J. Control. Release.* 101 (2005) 233–246. <https://doi.org/10.1016/j.jconrel.2004.06.023>.
- [99] C. V. Synatschke, A. Schallon, V. Jérôme, R. Freitag, A.H.E. Müller, Influence of Polymer Architecture and Molecular Weight of Poly(2-(dimethylamino)ethyl methacrylate) Polycations on Transfection Efficiency and Cell Viability in Gene Delivery, *Biomacromolecules.* 12 (2011) 4247–4255.  
<https://doi.org/10.1021/bm201111d>.
- [100] D. Santo, R.A. Cordeiro, A. Sousa, A. Serra, J.F.J. Coelho, H. Faneca, Combination of Poly[(2-dimethylamino)ethyl methacrylate] and Poly( $\beta$ -amino ester) Results in a Strong and Synergistic Transfection Activity, *Biomacromolecules.* 18 (2017) 3331–3342.  
<https://doi.org/10.1021/acs.biomac.7b00983>.
- [101] A.-K. Trützschler, T. Bus, M. Reifarth, J.C. Brendel, S. Hoepfener, A. Traeger, U.S. Schubert, Beyond Gene Transfection with Methacrylate-Based Polyplexes—The Influence of the Amino Substitution Pattern, *Bioconjug. Chem.* 29 (2018) 2181–2194. <https://doi.org/10.1021/acs.bioconjchem.8b00074>.
- [102] D.M. Lynn, R. Langer, Degradable Poly( $\beta$ -amino esters): Synthesis,

- Characterization, and Self-Assembly with Plasmid DNA, *J. Am. Chem. Soc.* 122 (2000) 10761–10768. <https://doi.org/10.1021/ja0015388>.
- [103] D.G. Anderson, A. Akinc, N. Hossain, R. Langer, Structure/Property Studies of Polymeric Gene Delivery Using a Library of Poly( $\beta$ -amino esters), *Mol. Ther.* 11 (2005) 426–434. <https://doi.org/10.1016/j.yymthe.2004.11.015>.
- [104] A. Akinc, D.G. Anderson, D.M. Lynn, R. Langer, Synthesis of Poly( $\beta$ -amino ester)s Optimized for Highly Effective Gene Delivery, *Bioconjug. Chem.* 14 (2003) 979–988. <https://doi.org/10.1021/bc034067y>.
- [105] J.-Y. Huang, Y. Gao, L. Cutlar, J. O’Keeffe-Ahern, T. Zhao, F.-H. Lin, D. Zhou, S. McMahon, U. Greiser, W. Wang, W. Wang, Tailoring Highly Branched Poly( $\beta$ -amino ester)s: a Synthetic Platform for Epidermal Gene Therapy, *Chem. Commun.* 51 (2015) 8473–8476. <https://doi.org/10.1039/C5CC02193F>.
- [106] S. Liu, Y. Gao, D. Zhou, M. Zeng, F. Alshehri, B. Newland, J. Lyu, J. O’Keeffe-Ahern, U. Greiser, T. Guo, F. Zhang, W. Wang, Highly Branched Poly( $\beta$ -amino ester) Delivery of Minicircle DNA for Transfection of Neurodegenerative Disease Related Cells, *Nat. Commun.* 10 (2019) 3307. <https://doi.org/10.1038/s41467-019-11190-0>.
- [107] Z. Zhong, Y. Song, J.F.J. Engbersen, M.C. Lok, W.E. Hennink, J. Feijen, A Versatile Family of Degradable Non-Viral Gene Carriers Based on Hyperbranched Poly(ester amine)s, *J. Control. Release.* 109 (2005) 317–329. <https://doi.org/10.1016/j.jconrel.2005.06.022>.
- [108] Y. Liu, Y. Li, D. Keskin, L. Shi, Poly( $\beta$ -Amino Esters): Synthesis, Formulations, and Their Biomedical Applications, *Adv. Healthc. Mater.* 8 (2018) 1801359. <https://doi.org/10.1002/adhm.201801359>.
- [109] R.A. Cordeiro, A. Serra, J.F.J. Coelho, H. Faneca, Poly( $\beta$ -amino ester)-Based

- Gene Delivery Systems: From Discovery to Therapeutic Applications, *J. Control. Release.* 310 (2019) 155–187. <https://doi.org/10.1016/j.jconrel.2019.08.024>.
- [110] Y. Cao, Y.F. Tan, Y.S. Wong, M.W.J. Liew, S. Venkatraman, Recent Advances in Chitosan-Based Carriers for Gene Delivery, *Mar. Drugs.* 17 (2019) 381. <https://doi.org/10.3390/md17060381>.
- [111] I. Younes, M. Rinaudo, Chitin and Chitosan Preparation from Marine Sources. Structure, Properties and Applications, *Mar. Drugs.* 13 (2015) 1133–1174. <https://doi.org/10.3390/md13031133>.
- [112] S.S. Lichtenberg, K. Nuti, J. DeRouche, O. V. Tsyusko, J.M. Unrine, Efficacy of Chitosan/Double-Stranded RNA Polyplex Nanoparticles for Gene Silencing Under Variable Environmental Conditions, *Environ. Sci. Nano.* 7 (2020) 1582–1592. <https://doi.org/10.1039/D0EN00137F>.
- [113] M. Mohammed, J. Syeda, K. Wasan, E. Wasan, An Overview of Chitosan Nanoparticles and Its Application in Non-Parenteral Drug Delivery, *Pharmaceutics.* 9 (2017) 53. <https://doi.org/10.3390/pharmaceutics9040053>.
- [114] M. Köping-Höggård, I. Tubulekas, H. Guan, K. Edwards, M. Nilsson, K. Vårum, P. Artursson, Chitosan as a Nonviral Gene Delivery System. Structure–Property Relationships and Characteristics Compared with Polyethylenimine In Vitro and After Lung Administration In Vivo, *Gene Ther.* 8 (2001) 1108–1121. <https://doi.org/10.1038/sj.gt.3301492>.
- [115] T. Kiang, J. Wen, H.W. Lim, K.W. Leong, The Effect of the Degree of Chitosan Deacetylation on the Efficiency of Gene Transfection, *Biomaterials.* 25 (2004) 5293–5301. <https://doi.org/10.1016/j.biomaterials.2003.12.036>.
- [116] J.-P. Nam, J.-W. Nah, Target Gene Delivery From Targeting Ligand Conjugated Chitosan–PEI Copolymer for Cancer Therapy, *Carbohydr. Polym.* 135 (2016)

- 153–161. <https://doi.org/10.1016/j.carbpol.2015.08.053>.
- [117] C. Xu, B. Yu, Y. Qi, N. Zhao, F. Xu, Versatile Types of Cyclodextrin-Based Nucleic Acid Delivery Systems, *Adv. Healthc. Mater.* 10 (2021) 2001183. <https://doi.org/10.1002/adhm.202001183>.
- [118] T. Azzam, H. Eliyahu, L. Shapira, M. Linial, Y. Barenholz, A.J. Domb, Polysaccharide–Oligoamine Based Conjugates for Gene Delivery, *J. Med. Chem.* 45 (2002) 1817–1824. <https://doi.org/10.1021/jm0105528>.
- [119] Y.-X. Sun, W. Xiao, S.-X. Cheng, X.-Z. Zhang, R.-X. Zhuo, Synthesis of (Dex-HMDDI)-g-PEIs as Effective and Low Cytotoxic Nonviral Gene Vectors, *J. Control. Release.* 128 (2008) 171–178. <https://doi.org/10.1016/j.jconrel.2008.03.004>.
- [120] J.J. Thomas, M.R. Rekha, C.P. Sharma, Unraveling the Intracellular Efficacy of Dextran-Histidine Polycation as an Efficient Nonviral Gene Delivery System, *Mol. Pharm.* 9 (2012) 121–134. <https://doi.org/10.1021/mp200485b>.
- [121] Y.. Park, Y.. Park, B.. Shin, E.. Choi, Y.. Park, T. Akaike, C.. Cho, Galactosylated Chitosan–graft–Dextran as Hepatocyte-Targeting DNA Carrier, *J. Control. Release.* 69 (2000) 97–108. [https://doi.org/10.1016/S0168-3659\(00\)00298-4](https://doi.org/10.1016/S0168-3659(00)00298-4).
- [122] M. Dréan, P. Guégan, C. Jérôme, J. Rieger, A. Debuigne, Far Beyond Primary Poly(vinylamine)s Through Free Radical Copolymerization and Amide Hydrolysis, *Polym. Chem.* 7 (2016) 69–78. <https://doi.org/10.1039/C5PY01325A>.
- [123] M. Dréan, P. Guégan, C. Detrembleur, C. Jérôme, J. Rieger, A. Debuigne, Controlled Synthesis of Poly(vinylamine)-Based Copolymers by Organometallic-Mediated Radical Polymerization, *Macromolecules.* 49 (2016) 4817–4827.

<https://doi.org/10.1021/acs.macromol.6b00992>.

- [124] M. Dréan, A. Debuigne, C. Goncalves, C. Jérôme, P. Midoux, J. Rieger, P. Guégan, Use of Primary and Secondary Polyvinylamines for Efficient Gene Transfection, *Biomacromolecules*. 18 (2017) 440–451. <https://doi.org/10.1021/acs.biomac.6b01526>.
- [125] M. Dréan, A. Debuigne, C. Jérôme, C. Goncalves, P. Midoux, J. Rieger, P. Guégan, Poly( N-methylvinylamine)-Based Copolymers for Improved Gene Transfection, *Macromol. Biosci.* 18 (2018) 1700353. <https://doi.org/10.1002/mabi.201700353>.
- [126] O. Boussif, F. Lezoualc'h, M. a Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A Versatile Vector for Gene and Oligonucleotide Transfer into Cells in Culture and In Vivo: Polyethylenimine, *Proc. Natl. Acad. Sci. U. S. A.* 92 (1995) 7297–7301. <https://doi.org/10.1073/pnas.92.16.7297>.
- [127] C. Gallops, J. Ziebarth, Y. Wang, A Polymer Physics Perspective on Why PEI Is an Effective Nonviral Gene Delivery Vector, in: *ACS Symp. Ser.*, American Chemical Society, 2020: pp. 1–12. <https://doi.org/10.1021/bk-2020-1350.ch001>.
- [128] E. Haladjova, S. Halacheva, V. Posheva, E. Peycheva, V. Moskova-Doumanova, T. Topouzova-Hristova, J. Doumanov, S. Rangelov, Comblike Polyethylenimine-Based Polyplexes: Balancing Toxicity, Cell Internalization, and Transfection Efficiency via Polymer Chain Topology, *Langmuir*. 31 (2015) 10017–10025. <https://doi.org/10.1021/acs.langmuir.5b02408>.
- [129] M.A. Cortez, W.T. Godbey, Y. Fang, M.E. Payne, B.J. Cafferty, K.A. Kosakowska, S.M. Grayson, The Synthesis of Cyclic Poly(ethylene imine) and Exact Linear Analogues: An Evaluation of Gene Delivery Comparing Polymer Architectures, *J. Am. Chem. Soc.* 137 (2015) 6541–6549.



<https://doi.org/10.1021/jacs.5b00980>.

- [130] I. Willner, Y. Eichen, A.J. Frank, M.A. Fox, Photoinduced Electron-Transfer Processes Using Organized Redox-Functionalized Bipyridinium-Polyethylenimine-Titania Colloids and Particulate Assemblies, *J. Phys. Chem.* 97 (1993) 7264–7271. <https://doi.org/10.1021/j100130a024>.
- [131] D. Wang, A.S. Narang, M. Kotb, A.O. Gaber, D.D. Miller, S.W. Kim, R.I. Mahato, Novel Branched Poly(Ethylenimine)–Cholesterol Water-Soluble Lipopolymers for Gene Delivery, *Biomacromolecules*. 3 (2002) 1197–1207. <https://doi.org/10.1021/bm025563c>.
- [132] B.-M. Jun, T.P.N. Nguyen, S.-H. Ahn, I.-C. Kim, Y.-N. Kwon, The Application of Polyethyleneimine Draw Solution in a Combined Forward Osmosis/Nanofiltration System, *J. Appl. Polym. Sci.* 132 (2015) n/a-n/a. <https://doi.org/10.1002/app.42198>.
- [133] J.D. Ziebarth, Y. Wang, Understanding the Protonation Behavior of Linear Polyethylenimine in Solutions through Monte Carlo Simulations, *Biomacromolecules*. 11 (2010) 29–38. <https://doi.org/10.1021/bm900842d>.
- [134] M. Tang, F. Szoka, The Influence of Polymer Structure on the Interactions of Cationic Polymers with DNA and Morphology of the Resulting Complexes, *Gene Ther.* 4 (1997) 823–832. <https://doi.org/10.1038/sj.gt.3300454>.
- [135] K. Itaka, A. Harada, Y. Yamasaki, K. Nakamura, H. Kawaguchi, K. Kataoka, In Situ Single Cell Observation by Fluorescence Resonance Energy Transfer Reveals Fast Intra-Cytoplasmic Delivery and Easy Release of Plasmid DNA Complexed with Linear Polyethylenimine, *J. Gene Med.* 6 (2004) 76–84. <https://doi.org/10.1002/jgm.470>.
- [136] B. Guillerm, S. Monge, V. Lapinte, J.J. Robin, How to Modulate the Chemical

- Structure of Polyoxazolines by Appropriate Functionalization, *Macromol. Rapid Commun.* 33 (2012) 1600–1612. <https://doi.org/10.1002/marc.201200266>.
- [137] C. Jiang, J. Chen, Z. Li, Z. Wang, W. Zhang, J. Liu, Recent Advances in the Development of Polyethylenimine-based Gene Vectors for Safe and Efficient Gene Delivery, *Expert Opin. Drug Deliv.* 16 (2019) 363–376. <https://doi.org/10.1080/17425247.2019.1604681>.
- [138] C. Diehl, H. Schlaad, Thermo-Responsive Polyoxazolines with Widely Tuneable LCST, *Macromol. Biosci.* 9 (2009) 157–161. <https://doi.org/10.1002/mabi.200800213>.
- [139] Y. Xie, N.H. Kim, V. Nadithe, D. Schalk, A. Thakur, A. Kılıç, L.G. Lum, D.J.P. Bassett, O.M. Merkel, Targeted Delivery of siRNA to Activated T Cells via Transferrin-Polyethylenimine (Tf-PEI) as a Potential Therapy of Asthma, *J. Control. Release.* 229 (2016) 120–129. <https://doi.org/10.1016/j.jconrel.2016.03.029>.
- [140] B. Pons, L. Mouhoubi, A. Adib, P. Godzina, J.-P. Behr, G. Zuber,  $\omega$ -Hydrazino Linear Polyethylenimine: A Monoconjugation Building Block for Nucleic Acid Delivery, *ChemBioChem.* 7 (2006) 303–309. <https://doi.org/10.1002/cbic.200500349>.
- [141] Z. Liu, Z. Zhang, C. Zhou, Y. Jiao, Hydrophobic Modifications of Cationic Polymers for Gene Delivery, *Prog. Polym. Sci.* 35 (2010) 1144–1162. <https://doi.org/10.1016/j.progpolymsci.2010.04.007>.
- [142] Y. Wang, M. Ye, R. Xie, S. Gong, Enhancing the In Vitro and In Vivo Stabilities of Polymeric Nucleic Acid Delivery Nanosystems, *Bioconjug. Chem.* 30 (2019) 325–337. <https://doi.org/10.1021/acs.bioconjchem.8b00749>.
- [143] N.P. Gabrielson, D.W. Pack, Acetylation of Polyethylenimine Enhances Gene

- Delivery via Weakened Polymer/DNA Interactions, *Biomacromolecules*. 7 (2006) 2427–2435. <https://doi.org/10.1021/bm060300u>.
- [144] S. Han, R.I. Mahato, S.W. Kim, Water-Soluble Lipopolymer for Gene Delivery, *Bioconjug. Chem.* 12 (2001) 337–345. <https://doi.org/10.1021/bc000120w>.
- [145] M. Thomas, A.M. Klibanov, Enhancing Polyethylenimine's Delivery of Plasmid DNA into Mammalian Cells, *Proc. Natl. Acad. Sci.* 99 (2002) 14640–14645. <https://doi.org/10.1073/pnas.192581499>.
- [146] G.J. Lee, T. Kim, Fluorination Effect to Intermediate Molecular Weight Polyethylenimine for Gene Delivery Systems, *J. Biomed. Mater. Res. Part A*. 107 (2019) 2468–2478. <https://doi.org/10.1002/jbm.a.36753>.
- [147] P. Wu, X. Luo, H. Wu, Q. Zhang, K. Wang, M. Sun, D. Oupicky, Combined Hydrophobization of Polyethylenimine with Cholesterol and Perfluorobutyrate Improves siRNA Delivery, *Bioconjug. Chem.* 31 (2020) 698–707. <https://doi.org/10.1021/acs.bioconjchem.9b00834>.
- [148] D. Meneksedag-Erol, R.B. KC, T. Tang, H. Uludağ, A Delicate Balance When Substituting a Small Hydrophobe onto Low Molecular Weight Polyethylenimine to Improve Its Nucleic Acid Delivery Efficiency, *ACS Appl. Mater. Interfaces*. 7 (2015) 24822–24832. <https://doi.org/10.1021/acsami.5b07929>.
- [149] P.Y. Teo, C. Yang, J.L. Hedrick, A.C. Engler, D.J. Coady, S. Ghaem-Maghami, A.J.T. George, Y.Y. Yang, Hydrophobic Modification of Low Molecular Weight Polyethylenimine for Improved Gene Transfection, *Biomaterials*. 34 (2013) 7971–7979. <https://doi.org/10.1016/j.biomaterials.2013.07.005>.
- [150] J. Valencia-Serna, C. Kucharski, M. Chen, R. KC, X. Jiang, J. Brandwein, H. Uludağ, siRNA-Mediated BCR-ABL Silencing in Primary Chronic Myeloid Leukemia Cells Using Lipopolymers, *J. Control. Release*. 310 (2019) 141–154.

- <https://doi.org/10.1016/j.jconrel.2019.08.018>.
- [151] A. Alshamsan, A. Haddadi, V. Incani, J. Samuel, A. Lavasanifar, H. Uludağ, Formulation and Delivery of siRNA by Oleic Acid and Stearic Acid Modified Polyethylenimine, *Mol. Pharm.* 6 (2009) 121–133. <https://doi.org/10.1021/mp8000815>.
- [152] A.W. Dunn, V. V. Kalinichenko, D. Shi, Highly Efficient In Vivo Targeting of the Pulmonary Endothelium Using Novel Modifications of Polyethylenimine: An Importance of Charge, *Adv. Healthc. Mater.* 7 (2018) 1800876. <https://doi.org/10.1002/adhm.201800876>.
- [153] W. Shen, H. Wang, Y. Ling-hu, J. Lv, H. Chang, Y. Cheng, Screening of Efficient Polymers for siRNA Delivery in a Library of Hydrophobically Modified Polyethyleneimines, *J. Mater. Chem. B.* 4 (2016) 6468–6474. <https://doi.org/10.1039/C6TB01929C>.
- [154] E. Bertrand, C. Gonçalves, L. Billiet, J.P. Gomez, C. Pichon, H. Cheradame, P. Midoux, P. Guégan, Histidinylated Linear PEI: a New Efficient Non-Toxic Polymer for Gene Transfer, *Chem. Commun.* 47 (2011) 12547. <https://doi.org/10.1039/c1cc15716g>.
- [155] B. Maury, C. Gonçalves, G. Tresset, M. Zeghal, H. Cheradame, P. Guégan, C. Pichon, P. Midoux, Influence of pDNA Availability on Transfection Efficiency of Polyplexes in Non-Proliferative Cells, *Biomaterials.* 35 (2014) 5977–5985. <https://doi.org/10.1016/j.biomaterials.2014.04.007>.
- [156] J.-P. Gomez, G. Tresset, C. Pichon, P. Midoux, Improved Histidinylated IPEI Polyplexes for Skeletal Muscle Cells Transfection, *Int. J. Pharm.* 559 (2019) 58–67. <https://doi.org/10.1016/j.ijpharm.2019.01.003>.
- [157] J.E. Ihm, I. Krier, J.M. Lim, S. Shim, D.K. Han, J.A. Hubbell, Improved

- Biocompatibility of Polyethylenimine (PEI) as a Gene Carrier by Conjugating Urocanic Acid: In Vitro and In Vivo, *Macromol. Res.* 23 (2015) 387–395. <https://doi.org/10.1007/s13233-015-3047-8>.
- [158] Q.-F. Zhang, C.-R. Luan, D.-X. Yin, J. Zhang, Y.-H. Liu, Q. Peng, Y. Xu, X.-Q. Yu, Amino Acid-Modified Polyethylenimines with Enhanced Gene Delivery Efficiency and Biocompatibility, *Polymers (Basel)*. 7 (2015) 2316–2331. <https://doi.org/10.3390/polym7111516>.
- [159] X. Chen, K. Xu, J. Yu, X. Zhao, Q. Zhang, Y. Zhang, Y. Cheng, Peptide Modified Polycations with pH Triggered Lytic Activity for Efficient Gene Delivery, *Biomater. Sci.* 8 (2020) 6301–6308. <https://doi.org/10.1039/D0BM01231A>.
- [160] J. Park, K. Singha, S. Son, J. Kim, R. Namgung, C.-O. Yun, W.J. Kim, A Review of RGD-Functionalized Nonviral Gene Delivery Vectors for Cancer Therapy, *Cancer Gene Ther.* 19 (2012) 741–748. <https://doi.org/10.1038/cgt.2012.64>.
- [161] S. Fu, X. Xu, Y. Ma, S. Zhang, S. Zhang, RGD Peptide-Based Non-Viral Gene Delivery Vectors Targeting Integrin  $\alpha v\beta 3$  for Cancer Therapy, *J. Drug Target.* 27 (2019) 1–11. <https://doi.org/10.1080/1061186X.2018.1455841>.
- [162] J.S. Suk, J. Suh, K. Choy, S.K. Lai, J. Fu, J. Hanes, Gene Delivery to Differentiated Neurotypic Cells with RGD and HIV Tat Peptide Functionalized Polymeric Nanoparticles, *Biomaterials*. 27 (2006) 5143–5150. <https://doi.org/10.1016/j.biomaterials.2006.05.013>.
- [163] Y.M. Lee, D. Lee, J. Kim, H. Park, W.J. Kim, RPM Peptide Conjugated Bioreducible Polyethylenimine Targeting Invasive Colon Cancer, *J. Control. Release.* 205 (2015) 172–180. <https://doi.org/10.1016/j.jconrel.2015.01.020>.
- [164] H. Yan, K. Tram, Glycotargeting to Improve Cellular Delivery Efficiency of

- Nucleic Acids, *Glycoconj. J.* 24 (2007) 107–123. <https://doi.org/10.1007/s10719-006-9023-y>.
- [165] D. Appelhans, H. Komber, M.A. Quadir, S. Richter, S. Schwarz, J. van der Vlist, A. Aigner, M. Müller, K. Loos, J. Seidel, K.-F. Arndt, R. Haag, B. Voit, Hyperbranched PEI with Various Oligosaccharide Architectures: Synthesis, Characterization, ATP Complexation, and Cellular Uptake Properties, *Biomacromolecules*. 10 (2009) 1114–1124. <https://doi.org/10.1021/bm801310d>.
- [166] D. Gutsch, D. Appelhans, S. Höbel, B. Voit, A. Aigner, Biocompatibility and Efficacy of Oligomaltose-grafted Poly(ethylene imine)s (OM-PEIs) for in Vivo Gene Delivery, *Mol. Pharm.* 10 (2013) 4666–4675. <https://doi.org/10.1021/mp400479g>.
- [167] P. Erbacher, T. Bettinger, P. Belguise-Valladier, S. Zou, J.-L. Coll, J.-P. Behr, J.-S. Remy, Transfection and Physical Properties of Various Saccharide, Poly(ethylene glycol), and Antibody-Derivatized Polyethylenimines (PEI), *J. Gene Med.* 1 (1999) 210–222. [https://doi.org/10.1002/\(SICI\)1521-2254\(199905/06\)1:3<210::AID-JGM30>3.0.CO;2-U](https://doi.org/10.1002/(SICI)1521-2254(199905/06)1:3<210::AID-JGM30>3.0.CO;2-U).
- [168] K. Kunath, A. von Harpe, D. Fischer, T. Kissel, Galactose-PEI–DNA Complexes for Targeted Gene Delivery: Degree of Substitution Affects Complex Size and Transfection Efficiency, *J. Control. Release*. 88 (2003) 159–172. [https://doi.org/10.1016/S0168-3659\(02\)00458-3](https://doi.org/10.1016/S0168-3659(02)00458-3).
- [169] I. Fajac, G. Thévenot, L. Bédouet, C. Danel, M. Riquet, M. Merten, C. Figarella, J.D. Ava-Santucci, M. Monsigny, P. Briand, Uptake of Plasmid/Glycosylated Polymer Complexes and Gene Transfer Efficiency in Differentiated Airway Epithelial Cells, *J. Gene Med.* 5 (2003) 38–48. <https://doi.org/10.1002/jgm.318>.
- [170] L.J.C. Albuquerque, A.C. Alavarse, M.C. Carlan da Silva, M.S. Zilse, M.T.

- Barth, I.C. Bellettini, F.C. Giacomelli, Sweet Vector for Gene Delivery: the Sugar Decoration of Polyplexes Reduces Cytotoxicity with a Balanced Effect on Gene Expression, *Macromol. Biosci.* 18 (2018) 1700299. <https://doi.org/10.1002/mabi.201700299>.
- [171] A. Dehshahri, R.K. Oskuee, M. Ramezani, Plasmid DNA Delivery into Hepatocytes Using a Multifunctional Nanocarrier Based on Sugarconjugated Polyethylenimine, *Gene Ther. Mol. Biol.* 14 (2012) 62–71.
- [172] A. Dehshahri, H. Sadeghpour, E. Mohazzabieh, S. Saatchi Avval, R. Mohammadinejad, Targeted double domain nanoplex based on galactosylated polyethylenimine enhanced the delivery of IL-12 plasmid, *Biotechnol. Prog.* 36 (2020) 1–11. <https://doi.org/10.1002/btpr.3002>.
- [173] H.J. Lee, J. Park, G.J. Lee, J.-M. Oh, T. Kim, Polyethylenimine-Functionalized Cationic Barley  $\beta$ -Glucan Derivatives for Macrophage RAW264.7 Cell-Targeted Gene Delivery Systems, *Carbohydr. Polym.* 226 (2019) 115324. <https://doi.org/10.1016/j.carbpol.2019.115324>.
- [174] A.K. Blakney, Y. Abdouni, G. Yilmaz, R. Liu, P.F. McKay, C.R. Bouton, R.J. Shattock, C.R. Becer, Mannosylated Poly(ethylene imine) Copolymers Enhance saRNA Uptake and Expression in Human Skin Explants, *Biomacromolecules.* 21 (2020) 2482–2492. <https://doi.org/10.1021/acs.biomac.0c00445>.
- [175] X. Guan, Z. Guo, L. Lin, J. Chen, H. Tian, X. Chen, Ultrasensitive pH Triggered Charge/Size Dual-Rebound Gene Delivery System, *Nano Lett.* 16 (2016) 6823–6831. <https://doi.org/10.1021/acs.nanolett.6b02536>.
- [176] J. Chen, Z. Guo, Z. Jiao, L. Lin, C. Xu, H. Tian, X. Chen, Poly(l-glutamic acid)-Based Zwitterionic Polymer in a Charge Conversional Shielding System for Gene Therapy of Malignant Tumors, *ACS Appl. Mater. Interfaces.* 12 (2020)

19295–19306. <https://doi.org/10.1021/acsami.0c02769>.

- [177] M.A. Islam, T. Park, B. Singh, S. Maharjan, J. Firdous, M.-H. Cho, S.-K. Kang, C.-H. Yun, Y. Choi, C.-S. Cho, Major Degradable Polycations as Carriers for DNA and siRNA, *J. Control. Release.* 193 (2014) 74–89. <https://doi.org/10.1016/j.jconrel.2014.05.055>.
- [178] M. Breunig, U. Lungwitz, R. Liebl, A. Goepferich, Breaking up the Correlation Between Efficacy and Toxicity for Nonviral Gene Delivery, *Proc. Natl. Acad. Sci.* 104 (2007) 14454–14459. <https://doi.org/10.1073/pnas.0703882104>.
- [179] J. Zhu, M. Qiao, Q. Wang, Y. Ye, S. Ba, J. Ma, H. Hu, X. Zhao, D. Chen, Dual-Responsive Polyplexes with Enhanced Disassembly and Endosomal Escape for Efficient Delivery of siRNA, *Biomaterials.* 162 (2018) 47–59. <https://doi.org/10.1016/j.biomaterials.2018.01.042>.
- [180] Y. Lei, J. Wang, C. Xie, E. Wagner, W. Lu, Y. Li, X. Wei, J. Dong, M. Liu, Glutathione-Sensitive RGD-Poly(ethylene glycol)-SS-Polyethylenimine for Intracranial Glioblastoma Targeted Gene Delivery, *J. Gene Med.* 14 (2013) n/a-n/a. <https://doi.org/10.1002/jgm.2726>.
- [181] V. Knorr, V. Russ, L. Allmendinger, M. Ogris, E. Wagner, Acetal Linked Oligoethylenimines for use as pH-Sensitive Gene Carriers, *Bioconjug. Chem.* 19 (2008) 1625–1634. <https://doi.org/10.1021/bc8001858>.
- [182] J. Che, A. Tao, S. Chen, X. Li, Y. Zhao, W. Yuan, Biologically Responsive Carrier-Mediated Anti-Angiogenesis shRNA Delivery for Tumor Treatment, *Sci. Rep.* 6 (2016) 35661. <https://doi.org/10.1038/srep35661>.
- [183] H.-L. Jiang, Y.-K. Kim, R. Arote, J.-W. Nah, M.-H. Cho, Y.-J. Choi, T. Akaike, C.-S. Cho, Chitosan-Graft-Polyethylenimine as a Gene Carrier, *J. Control. Release.* 117 (2007) 273–280. <https://doi.org/10.1016/j.jconrel.2006.10.025>.



- [184] Y.H. Kim, J.H. Park, M. Lee, Y.-H. Kim, T.G. Park, S.W. Kim, Polyethylenimine with Acid-Labile Linkages as a Biodegradable Gene Carrier, *J. Control. Release.* 103 (2005) 209–219. <https://doi.org/10.1016/j.jconrel.2004.11.008>.
- [185] H. Petersen, T. Merdan, K. Kunath, D. Fischer, T. Kissel, Poly(ethylenimine-co-lactamide-co-succinamide): A Biodegradable Polyethylenimine Derivative with an Advantageous pH-Dependent Hydrolytic Degradation for Gene Delivery, *Bioconjug. Chem.* 13 (2002) 812–821. <https://doi.org/10.1021/bc0255135>.
- [186] M.L. Forrest, J.T. Koerber, D.W. Pack, A Degradable Polyethylenimine Derivative with Low Toxicity for Highly Efficient Gene Delivery, *Bioconjug. Chem.* 14 (2003) 934–940. <https://doi.org/10.1021/bc034014g>.
- [187] L. Zhang, M. Yu, J. Wang, R. Tang, G. Yan, W. Yao, X. Wang, Low Molecular Weight PEI-Based Vectors via Acid-Labile Ortho Ester Linkage for Improved Gene Delivery, *Macromol. Biosci.* 16 (2016) 1175–1187. <https://doi.org/10.1002/mabi.201600071>.
- [188] G. Fang, F. Zeng, C. Yu, S. Wu, Low Molecular Weight PEIs Modified by Hydrazone-Based Crosslinker and Betaine as Improved Gene Carriers, *Colloids Surfaces B Biointerfaces.* 122 (2014) 472–481. <https://doi.org/10.1016/j.colsurfb.2014.07.007>.
- [189] S. Xu, M. Chen, Y. Yao, Z. Zhang, T. Jin, Y. Huang, H. Zhu, Novel Poly(ethylene imine) Biscarbamate Conjugate as an Efficient and Nontoxic Gene Delivery System, *J. Control. Release.* 130 (2008) 64–68. <https://doi.org/10.1016/j.jconrel.2008.04.025>.
- [190] Y. Zhang, J. Zhou, S. Ma, Y. He, J. Yang, Z. Gu, Reactive Oxygen Species (ROS)-Degradable Polymeric NanoplatforM for Hypoxia-Targeted Gene

- Delivery: Unpacking DNA and Reducing Toxicity, *Biomacromolecules*. 20 (2019) 1899–1913. <https://doi.org/10.1021/acs.biomac.9b00054>.
- [191] C.-K. Chen, P.-K. Huang, W. Law, C. Chu, N. Chen, L. Lo, Biodegradable Polymers for Gene-Delivery Applications, *Int. J. Nanomedicine*. 15 (2020) 2131–2150. <https://doi.org/10.2147/IJN.S222419>.
- [192] A. Kolate, D. Baradia, S. Patil, I. Vhora, G. Kore, A. Misra, PEG — A Versatile Conjugating Ligand for Drugs and Drug Delivery Systems, *J. Control. Release*. 192 (2014) 67–81. <https://doi.org/10.1016/j.jconrel.2014.06.046>.
- [193] X. Shuai, T. Merdan, F. Unger, M. Wittmar, T. Kissel, Novel Biodegradable Ternary Copolymers hy-PEI-g -PCL-b-PEG: Synthesis, Characterization, and Potential as Efficient Nonviral Gene Delivery Vectors, *Macromolecules*. 36 (2003) 5751–5759. <https://doi.org/10.1021/ma034390w>.
- [194] H. Petersen, P.M. Fechner, A.L. Martin, K. Kunath, S. Stolnik, C.J. Roberts, D. Fischer, M.C. Davies, T. Kissel, Polyethylenimine-graft-Poly(ethylene glycol) Copolymers: Influence of Copolymer Block Structure on DNA Complexation and Biological Activities as Gene Delivery System, *Bioconjug. Chem*. 13 (2002) 845–854. <https://doi.org/10.1021/bc025529v>.
- [195] H.-K. Nguyen, P. Lemieux, S. V Vinogradov, C.L. Gebhart, N. Guérin, G. Paradis, T.K. Bronich, V.Y. Alakhov, a V Kabanov, Evaluation of Polyether-Polyethyleneimine Graft Copolymers as Gene Transfer Agents, *Gene Ther*. 7 (2000) 126–138. <https://doi.org/10.1038/sj.gt.3301052>.
- [196] R.J. Smith, R.W. Beck, L.E. Prevette, Impact of Molecular Weight and Degree of Conjugation on the Thermodynamics of DNA Complexation and Stability of Polyethylenimine-graft-Poly(ethylene glycol) Copolymers, *Biophys. Chem*. 203–204 (2015) 12–21. <https://doi.org/10.1016/j.bpc.2015.04.005>.

- [197] X. Zhang, S.-R. Pan, H.-M. Hu, G.-F. Wu, M. Feng, W. Zhang, X. Luo, Poly(ethylene glycol)-block-Polyethylenimine Copolymers as Carriers for Gene Delivery: Effects of PEG Molecular Weight and PEGylation Degree, *J. Biomed. Mater. Res. Part A*. 84A (2008) 795–804. <https://doi.org/10.1002/jbm.a.31343>.
- [198] M. Ogris, S. Brunner, S. Schüller, R. Kircheis, E. Wagner, PEGylated DNA/Transferrin–PEI Complexes: Reduced Interaction with Blood Components, Extended Circulation in Blood and Potential for Systemic Gene Delivery, *Gene Ther.* 6 (1999) 595–605. <https://doi.org/10.1038/sj.gt.3300900>.
- [199] S. Schöttler, G. Becker, S. Winzen, T. Steinbach, K. Mohr, K. Landfester, V. Mailänder, F.R. Wurm, Protein Adsorption is Required for Stealth Effect of Poly(ethylene glycol)- and Poly(phosphoester)-Coated Nanocarriers, *Nat. Nanotechnol.* 11 (2016) 372–377. <https://doi.org/10.1038/nnano.2015.330>.
- [200] I. Hamad, A.C. Hunter, J. Szebeni, S.M. Moghimi, Poly(ethylene glycol)s Generate Complement Activation Products in Human Serum through Increased Alternative Pathway Turnover and a MASP-2-Dependent Process, *Mol. Immunol.* 46 (2008) 225–232. <https://doi.org/10.1016/j.molimm.2008.08.276>.
- [201] P. Zhang, F. Sun, S. Liu, S. Jiang, Anti-PEG Antibodies in the Clinic: Current Issues and Beyond PEGylation, *J. Control. Release.* 244 (2016) 184–193. <https://doi.org/10.1016/j.jconrel.2016.06.040>.
- [202] T. Ishida, H. Kiwada, Accelerated Blood Clearance (ABC) Phenomenon Upon Repeated Injection of PEGylated Liposomes, *Int. J. Pharm.* 354 (2008) 56–62. <https://doi.org/10.1016/j.ijpharm.2007.11.005>.
- [203] S. Abbina, A. Parambath, PEGylation and its Alternatives, in: *Eng. Biomater. Drug Deliv. Syst.*, Elsevier, 2018: pp. 363–376. <https://doi.org/10.1016/B978-0-08-101750-0.00014-3>.

- [204] M. Bauer, L. Tauhardt, H.M.L. Lambermont-Thijs, K. Kempe, R. Hoogenboom, U.S. Schubert, D. Fischer, Rethinking the Impact of the Protonable Amine density on Cationic Polymers for Gene Delivery: A Comparative Study of Partially Hydrolyzed Poly(2-ethyl-2-oxazoline)s and Linear Poly(ethylene imine)s, *Eur. J. Pharm. Biopharm.* 133 (2018) 112–121. <https://doi.org/10.1016/j.ejpb.2018.10.003>.
- [205] A.K. Blakney, G. Yilmaz, P.F. McKay, C.R. Becer, R.J. Shattock, One Size Does Not Fit All: The Effect of Chain Length and Charge Density of Poly(ethylene imine) Based Copolymers on Delivery of pDNA, mRNA, and RepRNA Polyplexes, *Biomacromolecules.* 19 (2018) 2870–2879. <https://doi.org/10.1021/acs.biomac.8b00429>.
- [206] G.-H. Hsiue, H.-Z. Chiang, C.-H. Wang, T.-M. Juang, Nonviral Gene Carriers Based on Diblock Copolymers of Poly(2-ethyl-2-oxazoline) and Linear Polyethylenimine, *Bioconjug. Chem.* 17 (2006) 781–786. <https://doi.org/10.1021/bc050317u>.
- [207] E. Haladjova, S. Halacheva, D. Momekova, V. Moskova-Doumanova, T. Topouzova-Hristova, K. Mladenova, J. Doumanov, M. Petrova, S. Rangelov, Polyplex Particles Based on Comb-Like Polyethylenimine/Poly(2-ethyl-2-oxazoline) Copolymers: Relating Biological Performance with Morphology and Structure, *Macromol. Biosci.* 18 (2018) 1700349. <https://doi.org/10.1002/mabi.201700349>.
- [208] S.-J. Gwak, C. Macks, S. Bae, N. Cecil, J.S. Lee, Physicochemical Stability and Transfection Efficiency of Cationic Amphiphilic Copolymer/pDNA Polyplexes for Spinal Cord Injury Repair, *Sci. Rep.* 7 (2017) 11247. <https://doi.org/10.1038/s41598-017-10982-y>.

- [209] C.-F. Wang, Y.-X. Lin, T. Jiang, F. He, R.-X. Zhuo, Polyethylenimine-Grafted Polycarbonates as Biodegradable Polycations for Gene Delivery, *Biomaterials*. 30 (2009) 4824–4832. <https://doi.org/10.1016/j.biomaterials.2009.05.053>.
- [210] F. He, C.-F. Wang, T. Jiang, B. Han, R.-X. Zhuo, Poly[(5-methyl-5-allyloxycarbonyl-trimethylene carbonate)-co-(5,5-dimethyl-trimethylene carbonate)] with Grafted Polyethylenimine as Biodegradable Polycations for Efficient Gene Delivery, *Biomacromolecules*. 11 (2010) 3028–3035. <https://doi.org/10.1021/bm1008525>.
- [211] A. Chakraborty, J.J. Martín Lasola, N. Truong, R.M. Pearson, Serum-Independent Nonviral Gene Delivery to Innate and Adaptive Immune Cells Using Immunoplexes, *ACS Appl. Bio Mater.* 3 (2020) 6263–6272. <https://doi.org/10.1021/acsabm.0c00761>.
- [212] L.W. Warriner, J.R. Duke, D.W. Pack, J.E. DeRouchey, Succinylated Polyethylenimine Derivatives Greatly Enhance Polyplex Serum Stability and Gene Delivery In Vitro, *Biomacromolecules*. 19 (2018) 4348–4357. <https://doi.org/10.1021/acs.biomac.8b01248>.
- [213] A. Zintchenko, A. Philipp, A. Dehshahri, E. Wagner, Simple Modifications of Branched PEI Lead to Highly Efficient siRNA Carriers with Low Toxicity, *Bioconjug. Chem.* 19 (2008) 1448–1455. <https://doi.org/10.1021/bc800065f>.
- [214] M. Cao, Y. Gao, N. Qiu, Y. Shen, P. Shen, Folic Acid Directly Modified Low Molecular Weight of Polyethyleneimine for Targeted pDNA Delivery, *J. Drug Deliv. Sci. Technol.* 56 (2020) 101522. <https://doi.org/10.1016/j.jddst.2020.101522>.
- [215] A. Guo, Y. Wang, S. Xu, X. Zhang, M. Li, Q. Liu, Y. Shen, D. Cui, S. Guo, Preparation and Evaluation of pH-Responsive Charge-Convertible Ternary

- Complex FA-PEI-CCA/PEI/DNA with Low Cytotoxicity and Efficient Gene Delivery, *Colloids Surfaces B Biointerfaces*. 152 (2017) 58–67. <https://doi.org/10.1016/j.colsurfb.2017.01.007>.
- [216] W. Guo, R.J. Lee, Receptor-Targeted Gene Delivery via Folate-Conjugated Polyethylenimine, *AAPS PharmSci*. 1 (1999) 20–26. <https://doi.org/10.1208/ps010419>.
- [217] H. Ren, L. Zhou, M. Liu, W. Lu, C. Gao, Peptide GE11–Polyethylene Glycol–Polyethylenimine for targeted Gene Delivery in Laryngeal Cancer, *Med. Oncol*. 32 (2015) 185. <https://doi.org/10.1007/s12032-015-0624-9>.
- [218] M. Liu, Z.H. Li, F.J. Xu, L.H. Lai, Q.Q. Wang, G.P. Tang, W.T. Yang, An Oligopeptide Ligand-Mediated Therapeutic Gene Nanocomplex for Liver Cancer-Targeted Therapy, *Biomaterials*. 33 (2012) 2240–2250. <https://doi.org/10.1016/j.biomaterials.2011.11.082>.
- [219] J.L. Wang, G.P. Tang, J. Shen, Q.L. Hu, F.J. Xu, Q.Q. Wang, Z.H. Li, W.T. Yang, A Gene Nanocomplex Conjugated with Monoclonal Antibodies for Targeted Therapy of Hepatocellular Carcinoma, *Biomaterials*. 33 (2012) 4597–4607. <https://doi.org/10.1016/j.biomaterials.2012.02.045>.
- [220] H. Sadeghpour, B. Khalvati, E. Entezar-Almahdi, N. Savadi, S. Hossaini Alhashemi, M. Raoufi, A. Dehshahri, Double Domain Polyethylenimine-Based Nanoparticles for Integrin Receptor Mediated Delivery of Plasmid DNA, *Sci. Rep.* 8 (2018) 6842. <https://doi.org/10.1038/s41598-018-25277-z>.
- [221] J.D. Ziebarth, D.R. Kennetz, N.J. Walker, Y. Wang, Structural Comparisons of PEI/DNA and PEI/siRNA Complexes Revealed with Molecular Dynamics Simulations, *J. Phys. Chem. B*. 121 (2017) 1941–1952. <https://doi.org/10.1021/acs.jpcc.6b10775>.

- [222] I.Y. Perevyazko, M. Bauer, G.M. Pavlov, S. Hoepfener, S. Schubert, D. Fischer, U.S. Schubert, Polyelectrolyte Complexes of DNA and Linear PEI: Formation, Composition and Properties, *Langmuir*. 28 (2012) 16167–16176. <https://doi.org/10.1021/la303094b>.
- [223] Y. Hu, Z. He, Y. Hao, L. Gong, M. Pang, G.P. Howard, H.-H. Ahn, M. Brummet, K. Chen, H. Liu, X. Ke, J. Zhu, C.F. Anderson, H. Cui, C.G. Ullman, C.A. Carrington, M.G. Pomper, J.-H. Seo, R. Mittal, I. Minn, H.-Q. Mao, Kinetic Control in Assembly of Plasmid DNA/Polycation Complex Nanoparticles, *ACS Nano*. 13 (2019) 10161–10178. <https://doi.org/10.1021/acsnano.9b03334>.
- [224] Z. Wang, T. Zhu, C. Qiao, L. Zhou, B. Wang, J. Zhang, C. Chen, J. Li, X. Xiao, Adeno-Associated Virus Serotype 8 Efficiently Delivers Genes to Muscle and Heart, *Nat. Biotechnol.* 23 (2005) 321–328. <https://doi.org/10.1038/nbt1073>.
- [225] E.S. Lisitsyna, T.-M. Ketola, E. Morin-Picardat, H. Liang, M. Hanzlíková, A. Urtti, M. Yliperttula, E. Vuorimaa-Laukkanen, Time-Resolved Fluorescence Spectroscopy Reveals Fine Structure and Dynamics of Poly(l-lysine) and Polyethylenimine Based DNA Polyplexes, *J. Phys. Chem. B*. 121 (2017) 10782–10792. <https://doi.org/10.1021/acs.jpcc.7b08394>.
- [226] L. Pigeon, C. Gonçalves, C. Pichon, P. Midoux, Evidence for Plasmid DNA Exchange After Polyplex Mixing, *Soft Matter*. 12 (2016) 7012–7019. <https://doi.org/10.1039/C6SM00575F>.
- [227] S. Hou, N. Ziebac, S.A. Wieczorek, E. Kalwarczyk, V. Sashuk, T. Kalwarczyk, T.S. Kaminski, R. Holyst, Formation and Structure of PEI/DNA Complexes: Quantitative Analysis, *Soft Matter*. 7 (2011) 6967. <https://doi.org/10.1039/c1sm05449j>.
- [228] K. Utsuno, H. Uludağ, Thermodynamics of Polyethylenimine-DNA Binding and

- DNA Condensation, *Biophys. J.* 99 (2010) 201–207.  
<https://doi.org/10.1016/j.bpj.2010.04.016>.
- [229] T.-M. Ketola, M. Hanzlíková, L. Leppänen, M. Raviña, C.J. Bishop, J.J. Green, A. Urtti, H. Lemmetyinen, M. Yliperttula, E. Vuorimaa-Laukkanen, Independent versus Cooperative Binding in Polyethylenimine–DNA and Poly(l-lysine)–DNA Polyplexes, *J. Phys. Chem. B.* 117 (2013) 10405–10413.  
<https://doi.org/10.1021/jp404812a>.
- [230] V. Mengarelli, L. Auvray, D. Pastré, M. Zeghal, Charge Inversion, Condensation and Decondensation of DNA and Polystyrene Sulfonate by Polyethylenimine, *Eur. Phys. J. E.* 34 (2011) 127. <https://doi.org/10.1140/epje/i2011-11127-3>.
- [231] I.C. Bellettini, S.J. Fayad, V.G. Machado, E. Minatti, Properties of Polyplexes Formed Through Interaction Between Hydrophobically-Modified Poly(ethylene imine)s and Calf Thymus DNA in Aqueous Solution, *Soft Matter.* 13 (2017) 2609–2619. <https://doi.org/10.1039/C6SM02835G>.
- [232] A. Akinc, M.A. Maier, M. Manoharan, K. Fitzgerald, M. Jayaraman, S. Barros, S. Ansell, X. Du, M.J. Hope, T.D. Madden, B.L. Mui, S.C. Semple, Y.K. Tam, M. Ciufolini, D. Witzigmann, J.A. Kulkarni, R. van der Meel, P.R. Cullis, The Onpatro Story and the Clinical Translation of Nanomedicines Containing Nucleic Acid-Based Drugs, *Nat. Nanotechnol.* 14 (2019) 1084–1087.  
<https://doi.org/10.1038/s41565-019-0591-y>.
- [233] J.E. Zuckerman, I. Gritli, A. Tolcher, J.D. Heidel, D. Lim, R. Morgan, B. Chmielowski, A. Ribas, M.E. Davis, Y. Yen, Correlating Animal and Human Phase Ia/Ib Clinical Data with CALAA-01, a Targeted, Polymer-Based Nanoparticle Containing siRNA, *Proc. Natl. Acad. Sci.* 111 (2014) 11449–11454.  
<https://doi.org/10.1073/pnas.1411393111>.



- [234] M.E. Davis, J.E. Zuckerman, C.H.J. Choi, D. Seligson, A. Tolcher, C.A. Alabi, Y. Yen, J.D. Heidel, A. Ribas, Evidence of RNAi in Humans from Systemically Administered siRNA via Targeted Nanoparticles, *Nature*. 464 (2010) 1067–1070. <https://doi.org/10.1038/nature08956>.
- [235] J. Chen, Z. Guo, H. Tian, X. Chen, Production and Clinical Development of Nanoparticles for Gene Delivery, *Mol. Ther. - Methods Clin. Dev.* 3 (2016) 16023. <https://doi.org/10.1038/mtm.2016.23>.