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Review article

Polyethylenimine-based non-viral gene delivery systems☆

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Abstract

Gene therapy has become a promising strategy for the treatment of many inheritable or acquired diseases that are currently considered incurable. Non-viral vectors have attracted great interest, as they are simple to prepare, rather stable, easy to modify and relatively safe, compared to viral vectors. Unfortunately, they also suffer from a lower transfection efficiency, requiring additional effort for their optimization. The cationic polymer polyethylenimine (PEI) has been widely used for non-viral transfection in vitro and in vivo and has an advantage over other polycations in that it combines strong DNA compaction capacity with an intrinsic endosomolytic activity. Here, we give some insight into strategies developed for PEI-based non-viral vectors to overcome intracellular obstacles, including the improvement of methods for polyplex preparation and the incorporation of endosomolytic agents or nuclear localization signals. In recent years, PEI-based non-viral vectors have been locally or systemically delivered, mostly to target gene delivery to tumor tissue, the lung or liver. This requires strategies to efficiently shield transfection polyplexes against non-specific interaction with blood components, extracellular matrix and untargeted cells and the attachment of targeting moieties, which allow for the directed gene delivery to the desired cell or tissue. In this context, materials, facilitating the design of novel PEI-based non-viral vectors are described.

Keywords: Polyethylenimine; PEI; Intracellular trafficking; Endosomolytic peptides; Nuclear targeting; Cell targeting

1. Introduction

In recent years, the knowledge of the molecular mechanisms of many inheritable or acquired diseases has been greatly expanded, directing great attention to the field of gene therapy [1–6]. Antisense, ribozyme strategies [7] or iRNA [8] could potentially be used to down-regulate or inactivate the expression of specific genes. In addition, suicide gene therapy [9] could enable the selective destruction of tumor cells using prodrug-converting enzymes and tumor specific promoters.

Viral vectors have been applied to deliver therapeutic genes into living cells, but their broad use is affected by the limited size of the genetic material that can be delivered and severe safety risks [10,11], based upon their immunogenicity and their oncogenic potential [12–14].

In light of these concerns, non-viral gene delivery has emerged as a promising alternative. Among the variety of different materials [15–18] which have been utilized in the manufacture of non-viral vectors, the use of polymers confers several advantages, due to their ease of preparation, purification and chemical modification as well as their enormous stability. The polyamine PEI has emerged as a potent candidate, even though the use of PEI-derived gene delivery vehicles is still limited by a relatively low transfection efficiency and short duration of gene expression [19,20] compared to viral transfection systems, as well as cytotoxic effects [21,22].

In view of the great diversity within the field of non-viral gene delivery, here we chose to focus on polyethylenimine-based transfection vehicles, including strategies for their optimization and the observed effects thereof.

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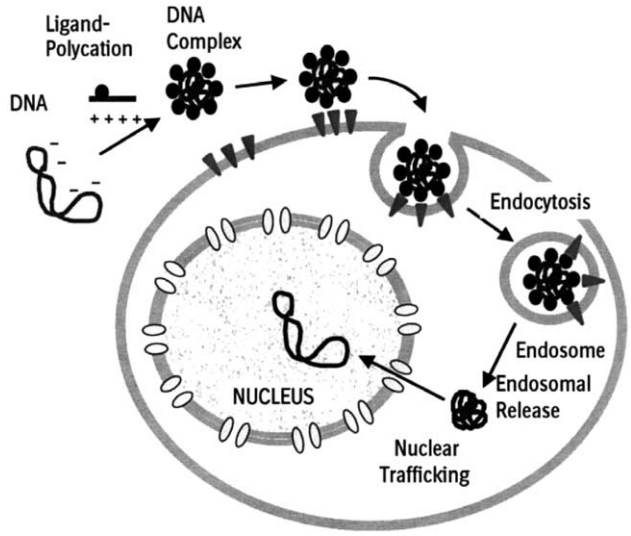


Fig. 1. General scheme of gene transfer using ligand-decorated non-viral vectors: DNA is condensed using the ligand–PEI conjugate to form ligand-decorated complexes. After their cellular uptake via receptor-mediated endocytosis, the polyplexes are entrapped in endosomes. The receptor–ligand complex dissociates from the PEI/DNA complex and the receptor is recycled to the cell surface. The polyplexes escape from endosomes into the cytoplasm and DNA crosses the nuclear envelope to accumulate in the nucleus. [Reprinted from J. Controll. Rel., 72 (2001), R. Kircheis, T. Blessing, S. Brunner, L. Wightman, E. Wagner, Tumor targeting with surface-shielded ligand–polycation DNA complexes, pp. 165–170, Copyright (2004) with permission from Elsevier].

2. Intracellular pathway

Despite the broad experimental use of PEI-based vectors, the efficiency of gene delivery remains insufficient. As understanding of the intracellular trafficking pathways has expanded, the major hurdles of gene transfer at the cellular level have been revealed, and the first strategies to overcome these limitations have been developed.

When considering the therapeutic use of non-viral gene transfer, cell internalization of exogenous DNA packed within polyplexes via receptor-mediated endocytosis remains the most favorable mechanism and can be accomplished by labeling the polyplexes with targeting moieties (Fig. 1). Unmodified cationic complexes interact non-specifically with negatively charged glycoproteins, proteoglycans and sulfated proteoglycans [23–26] located on the surface of cell membranes and enter the cell predominantly via adsorptive [27] or fluid-phase endocytosis [28]. The cell-polyplex association and, hence, the efficacy of cellular uptake can be greatly improved by increasing the positive net charge, prolonging the incubation time or raising the polyplex concentration [27,29,30]. Alternative paths of cell internalization include phagocytosis, particularly following particle aggregation at low *N*/*P* ratios (*N*/*P* ratio: quotient of the nitrogen atoms of PEI to DNA phosphates), and potocytosis (cellular uptake via interaction with caveolae pits [31]), but both depend largely on the cell type. Irrespective of the mechanism of endocytotic uptake, polyplexes will follow the endolysosomal pathway, remain closely attached to the vesicle membrane of early and late endosomes, and fuse with the lysosomal vesicles, assembling in the perinuclear region. The fusion of endosomes with lysosomes seems to be at least partially prevented when PEI has been used in polyplex preparation. The DNA is allowed to reside longer in the non-lysosomal compartment, thereby bypassing DNA degradation by lysosomal nucleases and promoting transfection [27,28,30]. Due to mechanisms, that are not yet fully elucidated, a sudden burst of endosomal vesicles occurs, liberating a detectable fraction of polyplexes into the cytoplasm and DNA is translocated into the nucleus [27].

It is still not clear whether or not intact polyplexes reside in the nuclear compartment. While Godbey et al. observed polyplexes within the nucleus, others could confirm these results only for necrotic cells, which lack an intact nuclear membrane [28,30]. For most non-viral vectors, a sufficient fraction of exogenous DNA reaches the transcription machinery only at the stage when the nuclear envelope breaks down [32], limiting efficient gene transfer to fast dividing cells.

One auspicious strategy uses protein transduction domains, such as the TAT protein of the HIV-1 virus [33] and herpes simplex virus type-1 VP22 transcription factor [34], to improve the efficacy of transgene delivery by simply circumventing the hurdles of the endocytotic pathway and simultaneously promoting translocation to the nucleus [35]. The positively charged protein transduction domains interact non-specifically with cell surfaces, promoting the internalization of larger cargo proteins directly into the cytoplasm in an endocytosis-independent manner. Optimized protein sequences appear to be attractive tools for use in the design of multifunctional and effective gene delivery vehicles and may conceivably reduce any immunogenic potential.

3. Polyethylenimine (PEI)

A large variety of different polymers and copolymers of linear, branched, and dendrimeric architecture, have been tested, in terms of their efficacy and suitability for in vitro transfection. Unfortunately, no morphology emerged as a general favorite [36]. The insight into the relationship between the polymer structure and their biological performance, such as the DNA compaction, toxicity and transfection efficiency is still rather limited. Hence, the discovery of new potent materials still relies on empiric approaches rather than on a rational design. Nonetheless, the results from transfection experiments with PEI were impressive from the beginning. Depending on the linkage of the repeating ethylenimine units, PEI occurs as branched or linear morphological isomers.

3.1. Branched PEI (bPEI)

bPEI-derived vectors have been used to deliver oligonucleotides [37], plasmid DNA (pDNA), and Epstein–Barr

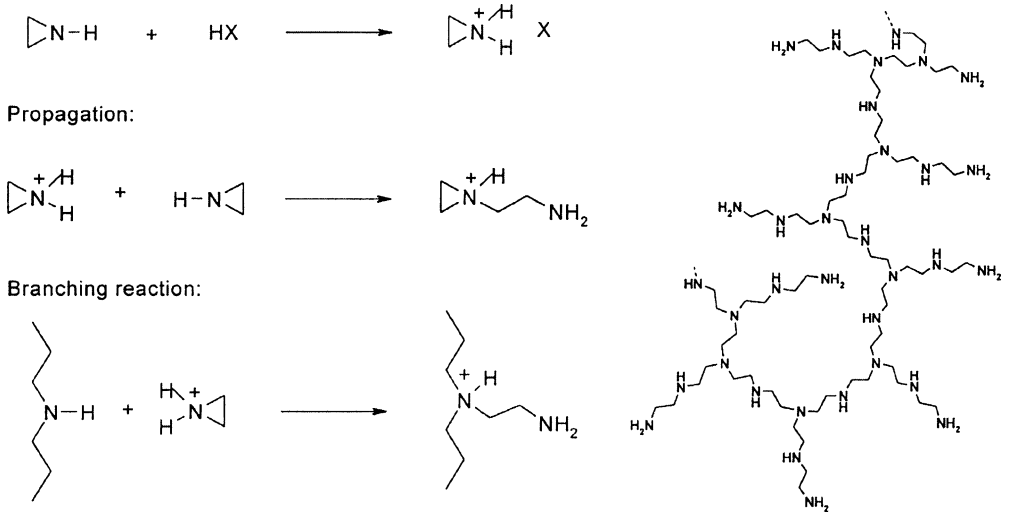


Fig. 2. Synthesis of branched polyethylenimine by acid catalyzed polymerization of aziridine in aqueous solution. [Reprinted from J. Controll. Rel., 69 (2000), A. von Harpe, H. Petersen, Y. Li, T. Kissel, Characterization of commercially available and synthesized polyethylenimine for gene delivery, pp. 309–322, Copyright (2004) with permission from Elsevier].

virus-based plasmid vectors [9] as well as RNA and intact ribozymes [7].

It is synthesized by us (Fig. 2) polymerization of aziridine either in aqueous [38–40] or alcoholic solutions, where the reaction is controlled by adjusting the temperature and initiator concentration, or in a rather vigorous bulk polymerization of anhydrous aziridine at a lower temperature [41,42].

The efficacy of bPEI-derived vectors non-viral vectors and their cytotoxic effects depend to a remarkable extent on material characteristics like the molecular weight, the degree of branching, the cationic charge density and buffer capacity [38–40], polyplex properties, such as the DNA content, particle size and zeta potential and the experimental conditions like the polyplex concentration, the presence or absence of serum during transfection, the incubation time and the transfection model chosen for the gene delivery experiment.

High molecular weight bPEI up to 800 kDa has been used for non-viral gene transfer, exhibiting a superior capability to form compact and stable bPEI/DNA complexes and an increased transfection efficiency, compared to lower molecular weight derivatives [43]. Unfortunately, at the same time the cell viability decreased remarkably. This effect can be moderated by transfecting with low molecular mass bPEIs (5–48 kDa) [39,40,44], but amplified polymer concentrations are needed to achieve comparable efficacy. Due to their reduced cytotoxicity, the high *N*/*P* ratios were tolerated and added to the superior performance in vitro, irrespective of the absence or presence of serum.

The *N*/*P* ratio, and with it the zeta potential, dramatically influences the efficacy of the gene delivery system. It has been estimated that every fifth or sixth amino nitrogen of bPEI is protonated at physiological pH [45] and only these positively charged amino groups will ionically interact with the negatively charged DNA. The pK_a value of the individual nitrogen atoms within the bPEI molecules cannot be determined and so the absolute amount of positively charged amino groups is not known. Therefore, the *N*/*P* ratio refers to the ratio of the nitrogen atoms of PEI to DNA phosphates and simply describes the amount of polymer used for polyplex formation. At high *N*/*P* ratios, the positive net charge of the corresponding complexes increases, improving cell interaction and enhancing the cellular and nuclear uptake and retention [46].

It has been shown that the relative gene expression can be strongly influenced by the particle size [47], which, in turn, can be moderated by the molecular weight of the bPEI derivative, the method of particle preparation, and the *N*/*P* ratio. The polyplex size decreases with increasing molecular weight, an excess of the polyamine and in low ionic strength media, most likely due to improved DNA compaction. The addition of proteins, from serum, for example, can additionally stabilize the newly formed particles. Unfortunately, the success of bPEI-based transfection systems is derogated by the cytotoxic effects, which arise from the presence of free polymer. The cytotoxic effects correlate with the molecular weight of the polymer and intranuclear polymer concentration [48] and increase with a prolonged incubation period. In vivo experiments have revealed that bPEI/DNA complexes and, to a lesser extent, free bPEI (25 kDa) activates genes involved in the Th1/Th2 immune response and adaptive immune responses [49]. The systemic administration of unshielded bPEI (800 kDa) had a lethal effect in animal models [50,51], demonstrating the need for materials allowing for the design of less harmful but effective non-viral vectors.

3.2. Linear PEI (lPEI)

More recently, several in vitro and in vivo studies have investigated the potential of lPEI-derived vectors. Most of these experiments have been done in direct comparison to the corresponding bPEI/DNA complexes, revealing remarkable differences between both transfection systems in terms of DNA compaction [19], nuclear uptake [32,52], transfection efficiency and toxicity [50,51,53].

lPEI has been synthesized via cationic ring-opening polymerization of either *N*(2-tetrahydropyranyl)azidirine [54] or unsubstituted and two-substituted 2-oxazolines followed by acid or base-catalyzed hydrolysis of the corresponding *N*-substituted polymer (Fig. 3) [46,55,56]. Potentiometric titration revealed that about 90% of the amines of the lPEI homopolymer are protonated at physiological pH. The suitability of lPEI for gene transfer was investigated and compared to random copolymers of poly(2-ethyl-2-oxazoline)-*co*-poly(ethylenimine) and *N*-alkylethylenimine, which are prepared by the partial hydrolysis or hydrogenation of the corresponding poly(2-ethyl-2-oxazoline) precursors, respectively [57,58]. As expected, increasing proportions of secondary amino groups within the random copolymers increased the buffer capacity and improved the DNA compaction, both contributing to the enhancement of transfection efficiency. The introduction of comparably less alkaline ternary amino groups dramatically decreased the transfection efficacy.

While bPEI/DNA-polyplexes retain a rather small size in the range up to a few hundred nanometers, which only slightly changes with respect to the complexation medium applied, lPEI mixed with pDNA forms rather large particles extending into the micrometer range in salt-containing buffers, which are prone to aggregation due to the weakened repulsion between the cationic particles [51,59]. Only preparation under salt-free conditions with increasing *N*/*P* ratios yields small spherical or toroid shaped particles, which grow rapidly upon the addition of salt. The application of small polyplexes has been shown to improve transgene expression up to 100-fold in vivo, but reduces the transfection efficiency in vitro, compared to the larger complexes [19,47,60]. While large particles sediment rapidly and therefore interact more with cell surfaces, the mobility of small particles is dictated by Brownian motion. This difference has been abrogated by decreasing the transfection volume or increasing the incubation time, both of which increase the chances of the smaller particles coming into contact with the cell surface. Another explanation for the comparably lower transgene expression mediated by small complexes in vitro has been attributed to the decreased endosomolytic competence [19,47]. Irrespective of the conditions used, lPEI/DNA complexes exhibited improved cell viability, promote nuclear localization and increased transfection efficiency compared to bPEI-based vectors [61].

4. Endosomolysis

PEI/DNA polyplexes are internalized by large variety of cells, delivering polymer/DNA complexes to the endolysosomal compartment. Advantageously in comparison to other polymers like poly(L-lysine), PEI combines a high membrane destabilizing potential with a high DNA condensing activity, protecting endocytosed DNA from degradation and therefore increasing the probability that intact pDNA will reach the nucleus [62,63].

Behr postulated the so-called 'proton sponge' hypothesis [25], which relates the intrinsic endosomolytic activity of

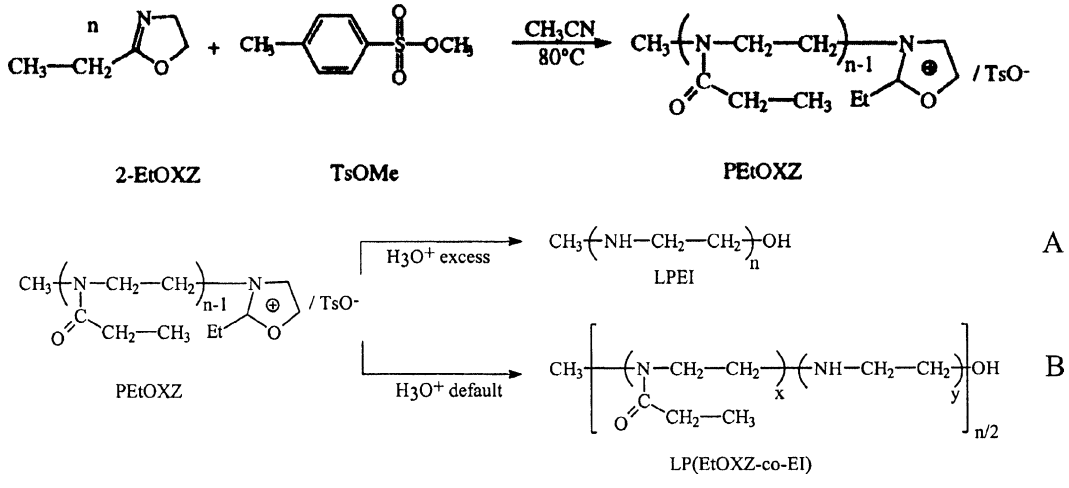


Fig. 3. Synthesis of linear polyethylenimine: the polymerization of 2-ethyl-2-oxazoline was initiated by methyl-*p*-toluenesulfonate and proceeds according to a living process, yielding poly(2-ethyl-2-oxazoline)[pOXZ], capped by oxazolinium tosylate ends. (A) Linear polyethylenimine was synthesized by complete hydrolysis of pOXZ, using an excess of hydrochloric acid in aqueous media. (B) The incomplete hydrolysis of pOXZ by addition of varying equivalents of hydrochloric acid, leads to the formation of random poly(2-ethyl-2-oxazoline-*co*-ethylenimine) copolymers. [Reprinted with permission from: Bioconjugate Chemistry, Vol. 14 (2003), pp. 581–587, B. Brissault, A. Kichler, Ch. Guis, Ch. Leborgne, O. Danos, H. Cheradame, Synthesis of Linear Polyethylenimine Derivatives for DNA Transfection, Copyright (2004) American Chemical Society].

PEI to its capacity to buffer the endosomal environment, prompting the osmotic swelling of the vesicle and finally its rupture, which leads to the liberation of the polyplexes into the cytoplasm. The fusion with lysosomes may thereby be prevented, circumventing DNA degradation by lysosomal DNases. In electron microscopy studies, membrane holes have been observed and were attributed to the direct interaction of bPEI with the inside of the endosomal membrane [30]. While acidification within endosomes has been suggested to facilitate vesicle rupture [64,65], likely due to the extension of the polymer network [62,66] the membrane damage occurred in a dose-dependent manner [48] and was present even in the absence of acidification when high molecular weight bPEI derivatives were used [30]. Furthermore, in vitro experiments using ^{35}S-DNA/bPEI complexes have revealed that the endosomolytic activity seems to depend on the particle size, suggesting that larger polyplexes entrapped in endosomes may facilitate endosomolysis [19,47,60,67], while the efficient gene delivery mediated by small particles relies on the addition of endosomolytic compounds [60].

Viruses like the adenovirus or influenza virus achieve cellular uptake by endocytosis and posses effective tools to promote endosomal escape [68]. Therefore, psoralen-inactivated adenoviruses were incorporated into bPEI/DNA polyplexes in the hopes to improving gene delivery. After systemic administration the relatively large particles were prone to phagocytosis by Kupffer cells and the subsequent immune response impeded a re-administration [69,70]. These systems have been simplified by attaching virus-derived endosomolytic proteins or optimized synthetic peptide sequences onto polyplexes, greatly reducing their immunogenic potential. JTS-derived fusogenic peptides [71,72], the *Haemophilus influenza* hemagglutinin-derived peptides [INF] [73–76] and GALA [74,77–80] all assume a random coil structure at pH 7. Acidification triggers a conformational transition, which enables the subsequent interaction with the phospholipid membranes, resulting in pore formation or the induction of membrane fusion and/or lysis [78]. The membrane-active dimeric influenza peptide INF5 ((GLF EAI EGF EIN GWEG nI DG)$_2$ K) [74] has been attached to both transferrin (Tf)-bPEI/DNA and bPEI/DNA polyplexes, enhancing transgene expression up to 10-fold [81]. However, the optimum expression levels observed were not significantly higher than the maximum expression at high *N*/*P* ratios without INF5. KALA (WEA KLA KAL AKA LAK HLA KAL AKA LKA CEA) [82,83] resembles the membranolytic activity of GALA (WEA ALA EAL AEA LAE HLA EAL AEA LEA LAA) [77,78] with DNA condensing properties derived from the lysine residues [83,84]. KALA has been site-specifically mono-PEGylated in order to preserve its fusogenic potential [82] and KALA and PEG–KALA were attached to the surface of bPEI/DNA polyplexes. The PEGylation sterically prevented particle aggregation and

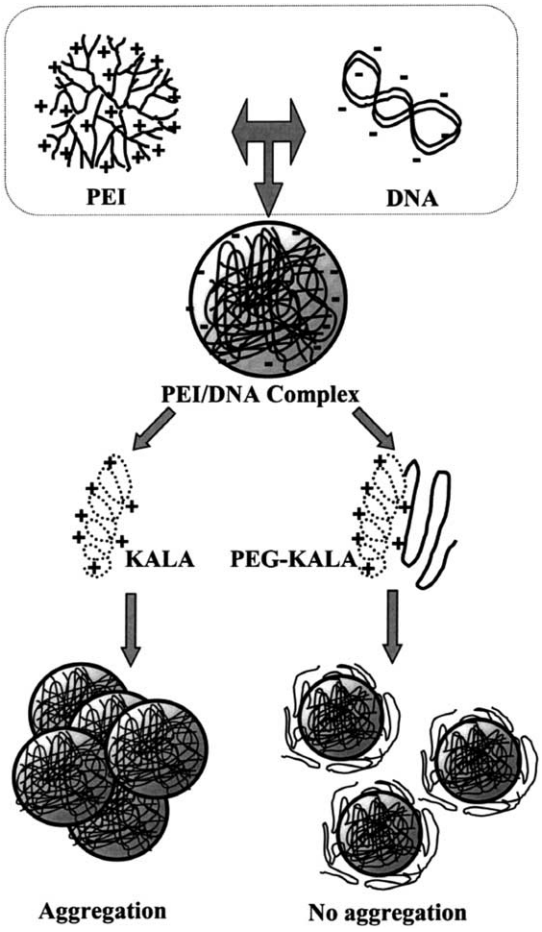


Fig. 4. The endosomolytic peptide KALA or a KALA–PEG conjugate was incorporated into PEI/DNA polyplexes by ionic interaction. While KALA–PEI/DNA increase significantly in size by inter-particle aggregation, pegylated polyplexes retain their individuality [Reprinted from J. Controll. Rel., 76 (2001), H. Lee, J.H. Jeong, T. G. Park, A new gene delivery formulation of polyethylenimine/DNA complexes coated with PEG-conjugated fusogenic peptide, pp. 183–192, Copyright (2004) with permission from Elsevier].

increasing concentrations of either bPEI or PEG–KALA led to an enhanced the transfection efficiency (Fig. 4).

However, the supplementation of membrane-destabilizing peptides, which is always inflicted with a rather low stability, the high costs of peptide synthesis and the risk of immunogenic reactions or reagents like chloroquine [85,86] failed to further enhance the transfection efficacy of PEI-based vectors, suggesting that the DNA liberation of PEI/DNA polyplexes into the cytoplasm is sufficiently supported by its own fusogenic activity.

5. Nuclear targeting

Successful gene therapy relies on an efficient DNA liberation from the endocytotic vesicles, DNA nuclear localization and gene expression, which proceeds in several steps, including: DNA nuclear localization, transcription factor nuclear import, transcription, mRNA processing

and export and finally, translation into the therapeutic protein [87]. After the release of DNA from endocytotic vesicles, pDNA larger than 2000 bp remains nearly immobile within the viscous cytoplasm [88], and is rapidly degraded by cytosolic nucleases [89,90]. PEI has been shown to prolong the survival of pDNA in a dose-dependent manner, facilitated by the tight complexation of DNA, without affecting nuclease activity itself [90,91]. Despite the broad use of PEI-based vectors, the precise mechanisms by which pDNA reaches the nuclear compartment remain elusive.

The double membrane of the nuclear envelope is perforated by nuclear pore complexes (NPC), which assembles 8 smaller diffusion channels to allow macromolecules with an upper size limit of 50 kDa to diffuse independently, controlled only by their size [92], and a large channel for signal-mediated transport of macromolecules larger than 50 kDa [93], which can expand to an upper diameter of 26 nm (8 million Da), depending on the species and the metabolic state of the cell (Fig. 5a and b) [94]. Therefore, the size [95] and copy number [96] of

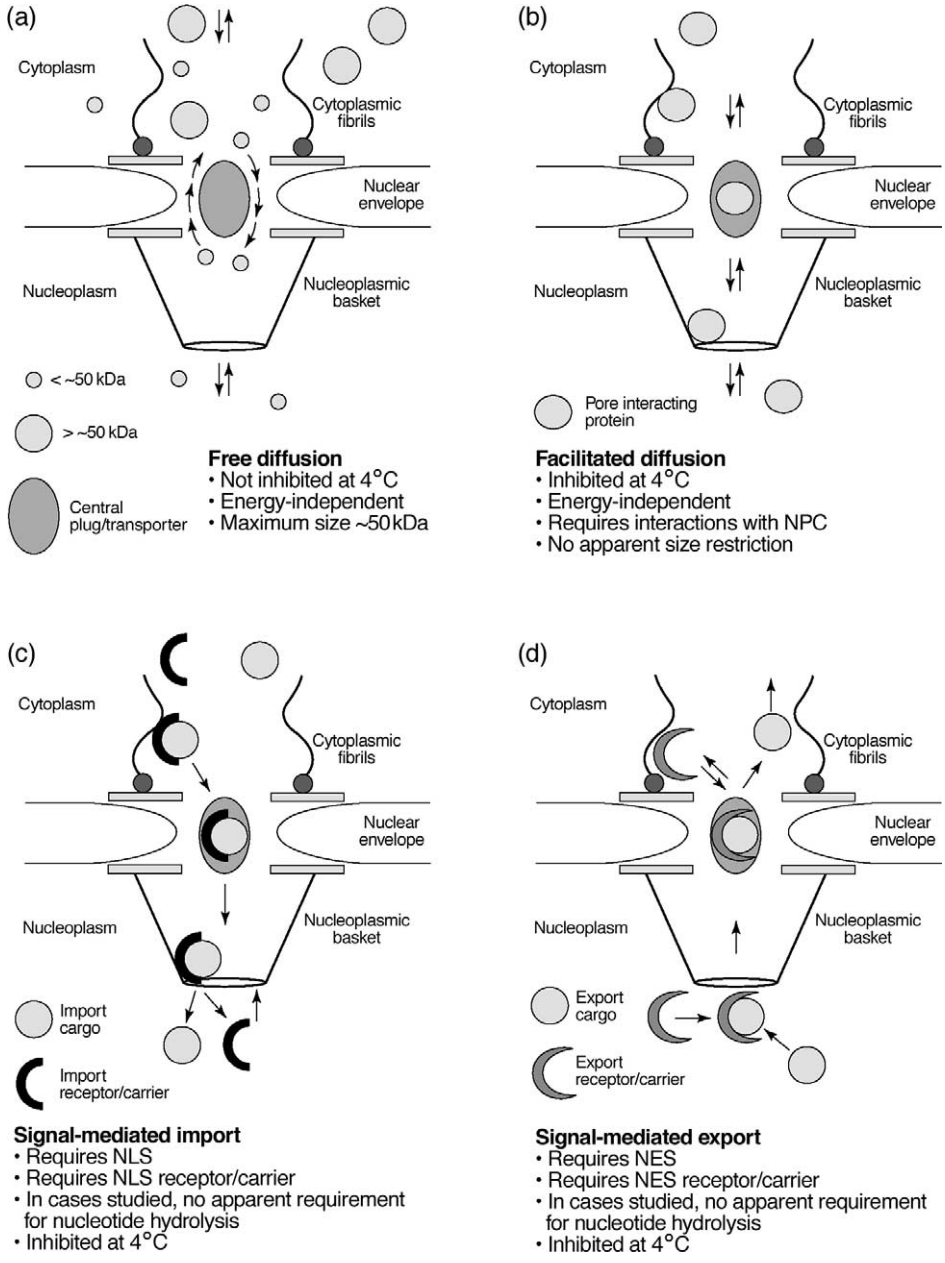


Fig. 5. Different pathways of macromolecule movement through the nuclear pore complex (NPC). (a) Macromolecules smaller than ≈50 kDa are able to diffuse freely through the NPC, macromolecules larger than ≈50 kDa cannot. (b) Nuclear transport carriers in the absence of cargo are thought to cross the NPC by facilitated diffusion. (c) Signal-mediated nuclear import: association of cargo and import carrier on the cytoplasmic side and transport through the NPC, dissociation at the nucleoplasm. (d) Signal-mediated nuclear export: assembly of cargo and export carrier at the nucleus, transport across the NPC and dissembly at the cytoplasma. [Reprinted from Trends in Cell Biology, 9 (1999), B. Talcott, M.S. Moore, Getting across the nuclear pore complex, pp. 312–318, Copyright (2004) with permission from Elsevier].

the pDNA largely influences the transfection efficiency. For active transport, substrates bind either directly to importin β or indirectly to importin α via nuclear localization signal-containing proteins to be carried through the nuclear pore [97–101] along the RanGDP/RanGTP gradient (Fig. 5c) [102,103].

The efficacy of transgene expression mediated by non-viral vectors, like bPEI/DNA polyplexes or, to a remarkably lesser extent, lPEI-derived systems, relies on the translocation of pDNA into the nucleus predominantly during the S/G2 phase of the cell cycle [32,52], which may explain why gene delivery is more efficient in rapidly dividing cells. As most cells targeted in gene therapy are non-dividing or divide very slowly, the incorporation of nuclear localization signals (NLS) that are capable of mediating nuclear import, [101,104,105] may conceivably both diminish the retention time of pDNA in the cytoplasm and facilitate the crossing of the nuclear barrier.

In several attempts, classical NLS peptides like the simian virus SV40 large T-antigen-derived peptides [106–112] and endogenous cellular proteins, such as the nucleoplasmin NLS [113], histones [93,114,115] or HMG-box proteins [93,116,117], as well as non-classical NLS, e.g. the HIV-1 virus TAT or REV peptides, the M9 sequence, derived from the heterogeneous nuclear ribonucleaprotein A1 [118,119] or SV40-DNA sequences [87,120–122], which interact with cytosolic transcription factors that harbor the desired NLS, have been applied to promote nuclear import. The latest approach is enticing because of the possibility of directing transgene expression to a specific cell [123] and then regulating it by the addition of exogenous stimulators [124]. The relative uptake and functional size of the channel can be influenced by the number of signals incorporated on each vector [125].

Other strategies have taken advantage of cytoplasmic glucocorticoid receptors, which carry cargo molecules across the nuclear envelope [126], or employed cytoplasmic transcription of the gene construct, alleviating the necessity for nuclear import [127]. In the latter, gene expression occurs immediately and proportionally to the amount of DNA released from endocytotic vesicles.

Zanta et al. prepared a CMVLuciferase-NLS gene containing a single, covalently linked SV40 large T-antigene NLS [PKKKRKVEDPYC] (Fig. 6) [128]. The luciferase reporter gene was capped on both ends with an ODN hairpin structure, increasing resistance to exonucleases up to 25-fold, and complexed to Transfectam®, bPEI 25 kDa or lPEI 22 kDa. The transfection efficiency of these vectors were enhanced 100–1000-fold even at DNA concentrations in the nanogram range, compared to polyplexes lacking the NLS peptide or prepared with a mutated NLS-peptide [PKTKRKVEDPYC]-conjugate. Here, the enhancement of transfection by nuclear targeting sequences seemed to be a general phenomenon, independent of the tissue origin or cell type.

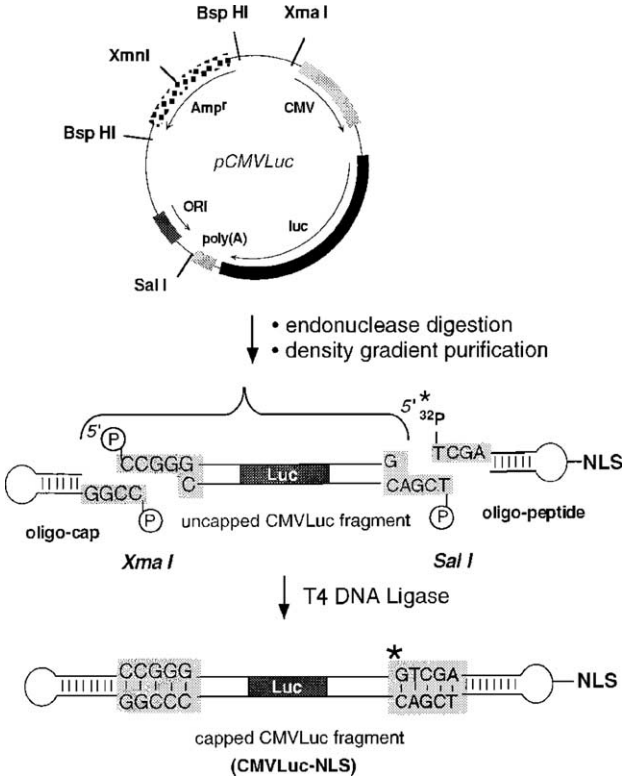


Fig. 6. Strategy for the preparation of a double-stranded DNA fragment coupled to an NLS peptide. A functional luciferase gene of 3380 bp was cut out of pCMVLuc with the restrictive endonucleases XmaI and SalI. Further digestion with XmnI and BspHI cut the unwanted restriction fragment into small fragments (970, 875, 768, and 240 bp) that were removed by sucrose gradient centrifugation. The capped CMVLuc-NLS DNA was obtained by ligation of the ^{32}P-labeled (*) oligonucleotide-peptide and oligonucleotide-cap hairpins to the restriction fragment. [M.A. Zanta, P. Belguise-Valladier, J.-P. Behr, Gene Delivery: A single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus, PNAS USA 96 (1999) 91–96. Copyright (2004) National Academy of Sciences, USA].

In contrast to the concept described above, Carlisle et al. [129] attached the adenovirus major capsid protein hexon covalently to bPEI 800 kDa via a disulfide or thioether bond and formed polyplexes with comparably larger plasmid DNA. In transfection experiments performed on HepG2 cells, hexon conjugates linked with disulfide bonds yielded the highest transfection efficiency, with up to 10–20-fold amplification over the control. Compared to classical NLS bearing conjugates, the hexon protein shows the strongest stimulation of nuclear entry.

It has recently been shown that lPEI/pDNA polyplexes disintegrate during their retention time within the cytoplasm, reaching a superior transfection capacity than the corresponding bPEI/pDNA complexes, which keep pDNA in its condensed state [130]. While for bPEI-derived vectors the attachment of NLS to prefabricated polyplexes may enhance nuclear entry by improving its accessibility for importin α-binding, lPEI-based gene delivery systems may benefit from strategies similar to that of Zanta et al. [128].

So far, the improvement of transgene delivery via NLS seems to be most pronounced for short oligonucleotides, while their impact on the transfection with pDNA remains rather low. The exclusion of pDNA from preformed nuclei after mitosis [131] or the nuclear export of exogenous DNA (Fig. 5d) [132] must also be considered, as these functions may limit the efficacy of non-viral transfection systems.

6. Applications

Polycations facilitate the tight compaction of pDNA into small and positively charged complexes, which are readily internalized via non-specific adsorptive endocytosis by a large variety of cells in vitro. In vivo, however, the specific delivery of highly charged particles to the desired cell fails, due to the non-specific interaction with blood components and extracellular matrix as well as non-targeted cells and tissues. During an incubation with human plasma, unmodified PEI/DNA polyplexes interact with IgD, IgM, proteins of the complement system C3β and C4γ, albumin, fibrinogen and apolipoproteins like apoA-I, A-II, H, C-III, and transthyretin [133,134]. The complement components adhere on the surface of complexes, triggering the activation of the complement system and removal by the reticulo-endothelial system (RES) [135]. The interaction with plasma proteins like albumin leads to the formation of ternary complexes, which tend to aggregate [47,136–139]. These large structures are rapidly cleared from the blood stream [140], most likely due to uptake by phagocytic cells or accumulation in fine capillary beds. This dramatically reduces the plasma circulation time and consequently limits the amount of therapeutic genes reaching the peripheral target cells and tissues. The aggregation with blood cells, especially erythrocytes [141,142], provokes the obstruction of blood vessels or lung capillaries [140,143], which may cause pulmonary embolism with potentially fatal consequences [47,140,143]. To suppress non-specific interactions, the cationic surface charge has been shielded by the covalent or non-covalent attachment of a hydrophilic polymer layer, using poly(ethylene glycol) (PEG), pluronic [139,144], polyacrylic acid (PAA) [145], poly(*N*-(2-hydroxypropyl)methacrylamide) derived copolymers [146,147], dextran [148] or dextran sulfates [149], as well as plasma proteins like transferrin [143,150] or human serum albumin [151]. It has been shown that shielded polyplexes remained soluble and small in size, and their susceptibility to salt-induced aggregation is drastically reduced. Once polyplexes extravasate into ambient tissue, they interact with the negatively charged components of the extracellular matrix, which may induce complex unraveling, exposing pDNA to degradation [152]. For PLL/DNA polyplexes, such side effects have been circumvented by cross-linking of the DNA condensing agent using intracellular reducible disulfide linkers. These nanogel-like particles, exhibited improved blood circulation times, increasing in parallel with the degree of cross-linking. Unfortunately, the efficacy of this transfection system suffered from the inefficient liberation of polyplexes into the cytoplasm, but this strategy may promote gene delivery in the case of vectors with a high intrinsic endosomolytic capacity, such as PEI-based vectors [153].

6.1. Polyplex shielding

Unfortunately, the large majority of copolymers used produce a shielding effect that counteracts effective DNA complexation, requiring a higher molecular weight of PEI, increased *N*/*P* ratios or cross-linking of the DNA-condensing polymer. Here, the most popular materials and optimized strategies for the shielding of PEI/DNA polyplexes will be described.

6.1.1. PEGylation

Linear PEG is a rather common biocompatible shielding reagent, widely used for drug delivery. The PEGylation of polyplexes has been achieved either by condensing DNA with PEG-PEI copolymers (pre-PEGylation) or coupling a PEG layer onto the surface of preformed polyplexes (post-PEGylation). Such pre- and post-PEGylated complexes can be concentrated by ultrafiltration, which is often required for the delivery of therapeutic amounts of DNA via systemic application [47,133,134,154] and can be stored at low temperatures until administration without changing the biophysical properties.

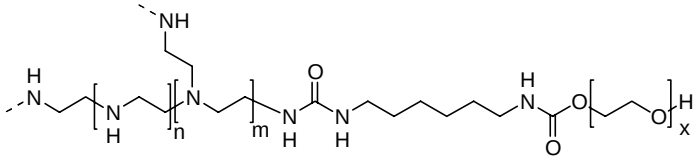
6.1.1.1. Pre-PEGylation. Several strategies have been used to prepare PEG–PEI copolymers, most of them using homobifunctional (Table 1: A1 and A2) or heterobifunctional PEG (Table 1: A3, A4 and C1) for conjugation onto branched or linear PEI [136–139,155,154]. Preserving one active group on the PEG terminus allows for the tagging a targeting molecule to the free PEG end (Table 1: A.5, A.6, A8 and C2) [133,134,156,157]. To enable the disintegration of the corresponding polyplexes and promote the DNA release at the cellular level, the shielding agent has been conjugated to the polycation by biodegradable disulfide bonds, which can be cleaved by intracellular reduction (Table 1: A7 and A8) [133].

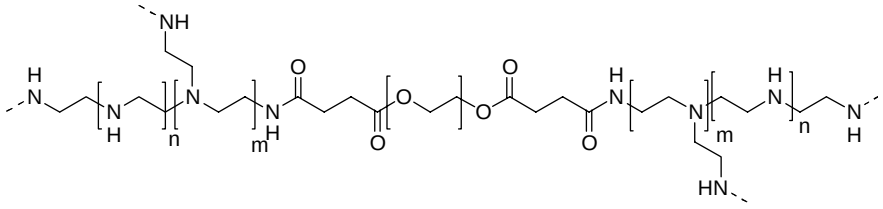
A major disadvantage of using copolymers for the preparation of shielded polyplexes is the reduced capacity of efficient DNA complexation [133,134,158]. This effect has been moderated by using higher molecular weight bPEI (800 kDa) [133,134] and longer PEG chains [141,142] for conjugation, by adding free bPEI or lPEI to the complexation medium [150] or using smaller ligands like EGF, instead of transferrin [133,134,158]. Higher PEG-grafting densities can prevent hemolysis or aggregation with erythrocytes [141,142], changing the biodistribution and gene expression pattern of these systems and likely aiding in an enhanced transfection efficiency in vivo [155].

Table 1
Materials for the manufacture of PEI/DNA polyplexes by application of PEI-derived copolymers or conjugation of a hydrophilic polymer layer onto the surface of preformed complexes

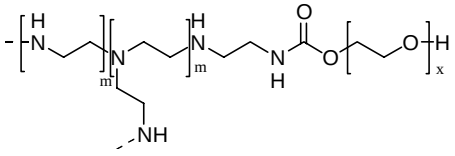
Pre-PEGylation

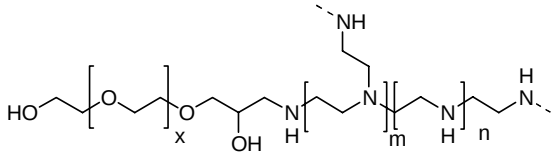
Conjugation by homobifunctional linker

A.1  [141,142]

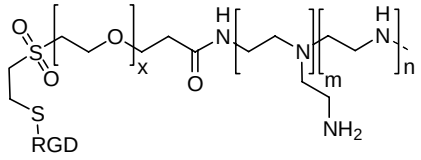
A.2  [136]

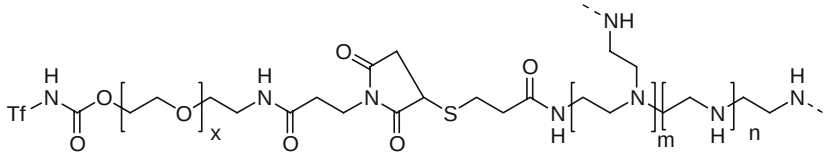
Conjugation by heterobifunctional PEG

A.3  [137,157]

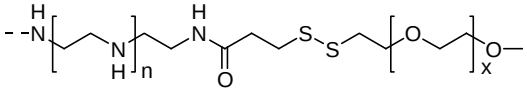
A.4  [139]

Containing a targeting moiety

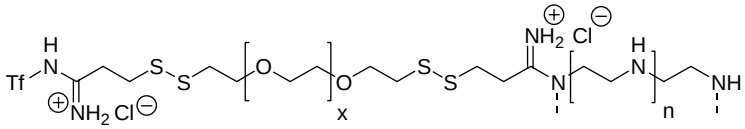
A.5  [156]

A.6  [134]

Copolymers cleavable by intracellular reduction

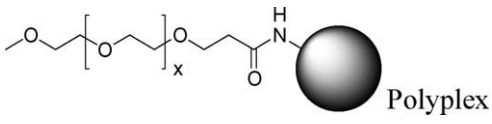
A.7 

Containing a targeting moiety

A.8 

Post-PEGylation

Conjugation of monofunctional PEG

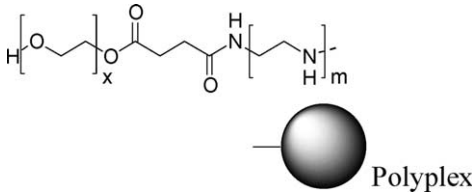
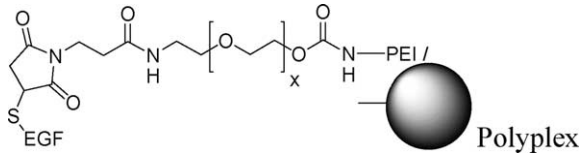
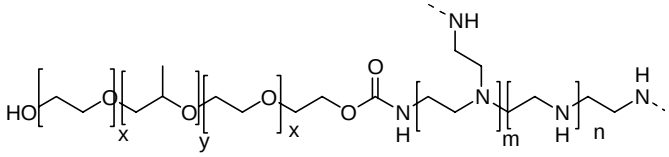
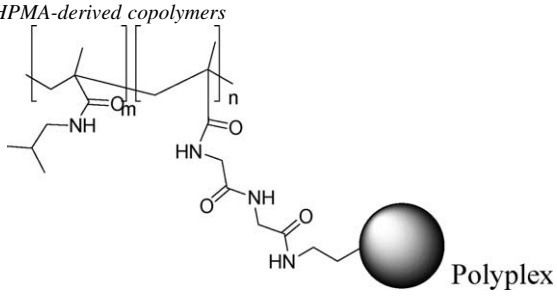
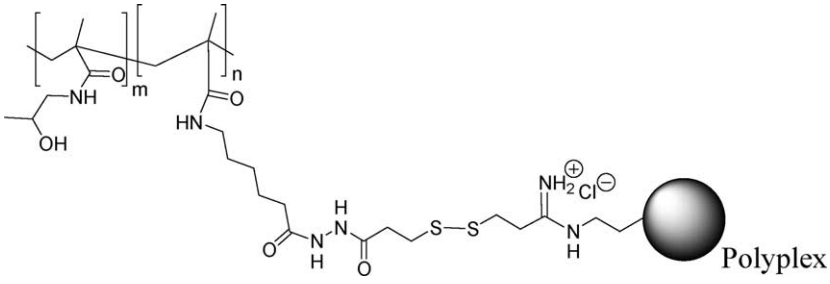
B.1  [134,140]

(*continued on next page*)

Pre/post-PEGylation

Table 1 (*continued*)

(*continued on next page*)

Conjugation by heterobifunctional PEG		
C.1		[154,157]
Containing a targeting moiety		
C.2		[133,138,155]
Pluronic P123-PEI-copolymer		
D.1		[139]
Coating of bPEI/pDNA polyplexes with pHPMA-derived copolymers		
E.1		[146]
E.2		[147]

A modified and rather elegant pre-PEGylation method led to the formation of shielded nanometer-sized polyplexes by mixing pDNA with prefabricated, purified and storable blends of ligand–PEG–PEI conjugates, PEG–PEI copolymers and PEI, forming a targeting unit, shielding agent, and DNA condensing agent, respectively. These transfection systems were based on either branched or linear PEI, shielded by linear or branched PEG derivatives of varying molecular weights. This method provides control over the particle properties, such as size and the extent of PEG shielding and ligand decoration, by the proportions of the single components within the mixture used for polyplex preparation (Fig. 7) [133,134].

6.1.1.2. Post-PEGylation. For post-PEGylation, the PEI/DNA complexes are prepared using unmodified PEI, ensuring that neither PEG nor the ligand competes with DNA compaction. The PEI/DNA polyplexes are usually formed in a solution with low ionic strength to generate small particles and the PEG chains are only allowed to react with the particle surface to create the protective PEG-shield.

The simplest strategy uses ligand-decorated PEI/DNA complexes and creates the protective layer by conjugating mono-activated PEG to the surface of the preformed polyplex (Table 1: B1) [133,140]. Here, the receptor ligand seems to be hidden beneath the PEG coating, impeding

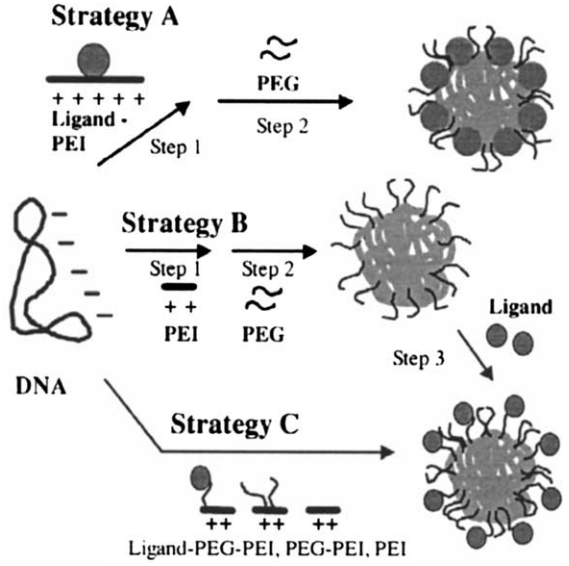


Fig. 7. Strategies for the PEGylation of PEI/DNA polyplexes: (A) a ligand–PEI conjugate is used to complex pDNA and the corresponding polyplex is pegylated in a second step. (B) PEI is used to condense pDNA. In a second step, PEG is conjugated to the polyplex surface. Finally, the ligand is attached to the distal ends of the PEG chains. (C) A mixture of ligand-decorated PEG–PEI copolymer, PEG–PEI copolymer and the homopolymer PEI is used to complex DNA, leading to the formation of PEG-shielded, ligand-decorated PEI/DNA polyplexes in a one step procedure. [Reprinted from J. Controll. Rel., 91 (2003), M. Ogris, G. Walker, T. Blessing, R. Kircheis, M. Wolschek, E. Wagner, Tumor-targeted gene therapy: strategies for the preparation of ligand–polyethylene glycol–polyethylenimine/DNA complexes, pp. 173–181, Copyright (2004) with permission from Elsevier].

the receptor binding and hence impairing cell internalization (Fig. 7).

In a more effective strategy, hetero-bifunctional PEG is attached to the surface amino groups of preformed unmodified PEI/DNA complexes. In the final synthesis step, a targeting moiety is conjugated to the distal ends of the PEG chains, ensuring maximum accessibility for receptor binding (Table 1: C1 and C2) [60,133,134].

In contrast to pre-PEGylation methods, post-PEGylation suffers from a rather time consuming sequence of conjugation steps that must be performed on DNA polyplexes. Therefore, the arsenal of plausible conjugation reactions and purification methods is limited and must be feasible at very low concentrations. Furthermore, the extent of PEG grafting or ligand tagging is challenging to control and characterize.

6.1.2. Pluronic

Pluronic P123-bPEI 2 kDa copolymers have been shown to form self-assembled micelle-like aggregates with an effective diameter of 60–70 nm, used to incorporate pDNA. The delivery of transgenes depended on the addition of free pluronic, which prevented polyplex precipitation in aqueous media. These transfection systems lead to a modulated biodistribution pattern and higher expression levels, compared to similar PEG–PEI/DNA complexes, an effect which is even more pronounced for low molecular weight bPEI (Table 1: D1) [139,144].

6.1.3. Polyacrylic acid

The addition of polyacrylic acid to preformed PEI/DNA polyplexes leads to the flocculation of neutrally charged ternary complexes of several hundred nanometers and drastically improved cell viability in vivo [145]. Comparative experiments using other polycarboxylic acids revealed that a sufficient anionic charge density is required to achieve effective charge shielding.

6.1.4. Poly(N-(2-hydroxypropyl)methacrylamide) [pHPMA]-derivatives

Oupicky et al. introduced pHPMA-derived copolymers for the coating of preformed bPEI25/DNA and bPEI800/DNA polyplexes. Advantageously in comparison to the rather common conjugation of monovalent PEG or pHPMA, the covering with multivalent pHPMA-derivatives protected the corresponding complexes from electrolyte exchange reactions and exhibited an improved plasma circulation performance with a significant localization in subcutaneously inoculated B16F10 tumors in C57BL/6 mice (Table 1: E1) [146]. The use of higher molecular weight pHPMA-derivatives and sufficient coating concentration have been shown to be the major prerequisite for an effective shielding. Unfortunately, these vectors showed a comparably low transfection activity, most likely due to the lack of intracellular dissociation and DNA release, which in turn inhibits efficient DNA transcription. Using biodegradable disulfide bonds to connect the shielding polymer to the PEI25/pDNA polyplex surface, allowed for the destabilization of the corresponding complexes after their reduction within the cytoplasm and nucleus. A coating extend of 20% was determined to be optimal, while higher substitution degrees reduced the transfection activity in vitro, most likely due to the depletion of the intracellular reduction capacity (Table 1: E2) [147].

6.1.5. Transferrin (Tf)

A very smart approach towards efficient polyplex shielding uses the plasma protein transferrin, which ideally combines both an intrinsic stealth effect and targeting towards transferrin-receptor expressing cells [143,150]. Applying blends of PEI and Tf-PEI (branched 25 kDa, 800 kDa or linear 22 kDa) yielded neutrally charged polyplexes at any *N/P* ratio and exhibited effective charge shielding by the incorporation of sufficient Tf ligand even in the absence of PEGylation. This gene delivery system not only exhibited decreased erythrocyte aggregation and therefore reduced toxicity even at high *N/P* ratios in vivo, but also shifted the biodistribution pattern from liver and lung, as usually observed for the unshielded complexes, to transgene expression predominantly in the targeted tumor.

6.2. Local application

The local application of non-viral vectors limits gene delivery to accessible target sites and depends on small, soluble and neutrally charged [59] polyplexes, that are capable of diffusing through tissue to reach their final destination. Even though the harsh environment of systemic blood circulation can be bypassed, complex unraveling due to the exposure to the extracellular matrix [152] and the risk of inflammatory and immune reactions [135] still have to be considered.

6.2.1. Lung

The mucus layer secreted by goblet cells, the almost impervious epithelium with tight junctions inhibiting the intercellular transport and the clearance by alveolar macrophages remain the major barriers for efficient PEI-based gene delivery targeted to the lung. In contrast to other non-viral transfection systems, the exposure to surfactant proteins [159] increased transgene expression by overcoming insufficient spreading of the polyplex solution on lung epithelia, while, the polyplex stability seemed to be unaffected [160]. The coadministration of penetration enhancers improved gene delivery across the dense epithelium, but unfortunately decreased the cell viability [161] as well. Intranasal application of lPEI22/DNA polyplexes has been shown to be superior to bPEI25/DNA vectors, yielding higher transgene expression. [53]. PEG-shielding of PEI/DNA polyplexes facilitated a homogenous gene expression pattern [162], but concomitantly caused drastically reduced transfection efficiencies [154], which seemed to be recovered only by the conjugation of a targeting moiety [162].

The more convenient aerosol delivery of unmodified bPEI25–DNA polyplexes subsequently transfected most of the epithelial cells in the conducting and peripheral airways. Gene expression was detectable up to 28 days after a single aerosol dose and increased with prolonged aerosol exposure [163].

6.2.2. Tumor

The most direct approach to achieve the expression of therapeutic genes in tumor tissue may be the injection of the polyplex solution into the tumor foci [9,143] or peritoneum to reach intraperitoneal disseminating tumors [164]. Labeling of bPEI/DNA complexes with receptor–ligand transferrin could thereby enhance the gene expression in tumor cells, due to the efficient internalization of the transfecting complexes into the tumor cells via receptor-mediated endocytosis [165].

For inaccessible tumors, polyplexes could be administered into the arterial blood vessels supplying the tumor. Polyplexes reach interstitial tumor tissue through irregular endothelial fenestrations. The enhanced permeation, the lack of normal lymphatic drainage and the hypervasculature of the tissue all lead to the accumulation of the complexes in the tumor interstitium (enhanced permeation and retention [EPR] effect).

6.2.3. Brain

The blood–brain barrier tends to preclude vascular delivery, as the non-fenestrated capillary endothelial cells surrounded by tight junctions control and limit the uptake of exogenous material. The transcellular movement by fluid phase endocytosis, which otherwise aids in the extravasation and diffusion of transgenes through the tissue, does not occur here. Gene delivery to the brain has been accomplished, however, by injection of lPEI22/DNA polyplexes into the cerebrospinal fluid or the lateral ventricle [166,167]. Polyplexes remained highly diffusible, spreading widely from the site of injection, distributing gene expression within both sides of the brain [59]. The intrathecal administration of a single dose of bPEI25/DNA into the lumbar subarachnoid space provided enhanced transgene expression in the spinal cord that was enhanced up to 40-fold, compared to naked DNA [155]. Unfortunately after the often required repeated application a up to 70% attenuation of gene expression was observed, which was associated with apoptotic cell death. By using shielded polyplexes prepared with a 1:1 PEG2–PEI25 conjugate, cell viability was improved, yielding 11-fold higher expression in the spinal cord, compared to the intrathecally injected homopolymer [138]. Another approach employed the capability of neurons to ingest exogenous material from the muscles they innervate. Hence, PEI/DNA complexes have been injected into the tongue, achieving retrograde axonal transport to hypoglossal motoneurons of the brain stem [168].

6.3. Systemic administration and receptor targeting

In order to reach distant organs or tumors, especially metastatic noodles, the systemic application of the therapeutic agent through the blood circulation should provide several advantages over the local administration. The directing of therapeutic systems to the targeted cell or tissue can be accomplished by simply taking advantage of special physiological conditions, e.g. the irregular fenestration in the liver, spleen, bone marrow or certain tumors, which facilitates passive accumulation. After intravenous [i.v.] injection, both unmodified lPEI and bPEI-derived vectors deliver the transgene into the heart, spleen, liver and kidney, with highest gene expression levels predominantly in the vascular endothelial cells of the lung, most likely due to the accumulation within the fine lung capillary beds [169,170]. But employing this organ tropism to direct gene delivery toward the lung via systemic administration seems to be only of little benefit, due to the reduced half-life, caused by the clearance of polyplexes from the systemic circulation after i.v. injection [171]. At increasing *N*/*P* ratios, gene expression levels could be enhanced in all organs, but the biodistribution pattern largely depended on

the method of polyplex administration and material characteristics. While bPEI25/DNA polyplexes promote the transfection of bronchial cells, lPEI22/DNA vectors yield the highest gene expression levels in the distal tract of the bronchial tree [172]. In contrast to lPEI/DNA complexes, with which transfection proceeded without any sign of severe toxicity, lung embolism and death of laboratory animals were observed after i.v. injection of bPEI25 and bPEI800/DNA polyplexes [51].

Receptor-targeting via the incorporation of receptor ligands like carbohydrates, transferrin, folate, growth factors, ligands for the low density lipoprotein receptor [173,174] or antibodies into the polyplex seems to be most favorable means to achieve site-directed gene delivery towards a preferred or specific cell type. An effective charge shielding is necessary to suppress competing internalization via adsorptive endocytosis, which can be more or less pronounced, due to the cell-specific presence of proteoglycans on the cell surface.

6.3.1. Glycosylated vehicles

Directing the therapeutic genes to hepatocytes or parenchymal liver cells may become a promising tool to correct genetic defects, contributing to the treatment of diseases like α1-antitrypsin deficiency, hemophilia and lipoprotein receptor deficiency, as well as cirrhosis or cancer. The asialoglycoprotein receptor (ASGPr), abundantly expressed in hepatocytes, internalizes galactose-terminated glycoproteins and *N*-acetylglucosamine residues via clathrin-coated pits [175]. To enhance the uptake into liver cells, mono- and oligosaccharides with an affinity for the ASGPr-receptor, like galactose [176–180], lactose [181,182] and larger carbohydrates like asialooromucoid [183,184], have been used for liver targeting, as they have a low immunogenicity compared to antibodies or peptides. The rate of cellular uptake varies between parenchymal and non-parenchymal cells and can be directed by the type of sugar conjugated to the gene delivery vehicle.

Galactose linked to 5% of the bPEI amino groups via a four-carbon hydrophilic spacer has been shown to direct gene transfer into hepatocytes highly selectively via ASGPr-receptor-mediated endocytosis [179,180]. Higher substitution degrees failed to achieve comparable transfection efficiencies, most likely due to decreased DNA compaction and hence lower polyplex stability [180]. This effect can be moderated by applying a higher molecular weight bPEI or increasing *N*/*P* ratios [176], but may drastically reduce the selectivity and efficacy of the gene transfer, which relies on neutrally charged complexes. The conjugation of linear tetragalactose at a grafting degree of 5% resulted in effective charge shielding, even at higher *N*/*P* ratios and the efficacy of gene delivery was further enhanced, most likely due to the improved particle stability and receptor accessibility [178]. When using PEG for charge shielding and ligand presentation, a grafting degree of 1% to bPEI25 has been shown to be optimal [177]. The transgene expression levels increased compared to unmodified PEI/DNA polyplexes, irrespective of the *N*/*P* ratio.

Mannose has been used to address gene delivery to dendritic cells [185], which express high levels of mannose receptors, mediating the internalization of mannosylated antigens [186]. Dendritic cells are capable of capturing antigens and provoking antigen-specific T cell responses by presenting the processed antigens under participation of the major histocompatibility complex (MHC) class I and II molecules [187]. Since several tumor cell-specific antigens have been identified and cloned [188], the tools are available to program dendritic cells to induce strong anti-tumor immune responses. Hence, dendritic cells represent an attractive cell type for immunotherapy, for example, for the treatment of cancer [189,190]. 1:1 Mannose–bPEI25 conjugates were prepared and used for DNA complexation. The gene delivery was augmented in a receptor-mediated manner, compared to unmodified bPEI/DNA polyplexes, which exhibited 50% less transgene expression. The incorporation of inactivated adenovirus particles further improved transfection efficiency up to 100-fold [185].

6.3.2. Transferrin receptor targeting

The transferrin receptor facilitates the uptake of transferrin [Tf]–iron complexes into erythrocytes as well as actively proliferating cells and rapidly dividing tissues, such as tumors. Transferrin, a serum glycoprotein, is conjugated to polyplexes by reductive amination via its carbohydrate residue, prompting its use as a targeting moiety to direct gene transfer, especially to tumor cells. It has been shown that the incorporation of transferrin into PEI/DNA polyplexes enhances the cell internalization via receptor-mediated endocytosis [81,191] and directs gene delivery efficiently to tumor tissue in vivo, even at low *N*/*P* ratios [133,134,140,143,150,192]. At higher grafting densities, transferrin itself exhibits effective charge shielding and therefore provides both polyplex protection and targeting [150]. The most efficient gene delivery into tumors was achieved with vectors prepared by complexing pDNA with a blend of transferrin tagged PEG–lPEI copolymer, PEG–lPEI copolymer and lPEI. The transgene expression was enhanced over equivalent systems derived from bPEI, transferrin-shielded particles and Tf-decorated PEG–bPEI/DNA complexes [133,134]. The gene expression levels varied between different tumors, due to deviant tumor vascularization, the occurrence of necrotic tissue and the infiltration by macrophages [192]. A significant fraction of polyplexes were taken up by macrophages, leading to a significant amount of DNA-degradation within the Kupffer cells of the liver [150].

The level of the firefly luciferase gene expression and the biodistribution pattern of transferrin-tagged polyplexes after systemic administration have been determined in living mice, using a cooled charge coupled device camera (Fig. 8) [193]. To enhance the intensity of the bioluminescence

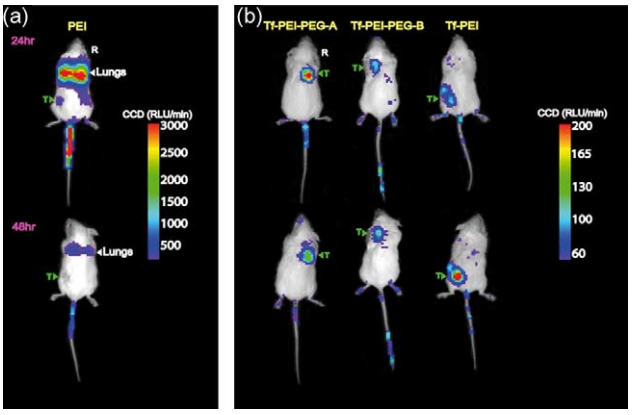


Fig. 8. Bioluminescent imaging of the optical reporter gene expression (fl: firefly luciferase gene) was determined and quantified in living, N2A tumor bearing mice using a cooled charge coupled device (CCD) camera. (a) LPEI22/DNA polyplexes were systemically injected into the mouse, resulting in much higher fl expression in the lung, compared with the tumor. (b) DNA was complexed using mixtures of transferrin labeled PEG-BPEI25: LPEI22-PEG20 (Tf-PEI-PEGA), transferrin-PEG-BPEI25: LPEI22-PEG40 (Tf-PEI-PEGB) or transferrin-BPEI25. The highest reporter gene expression was observed in the tumor and tail vain, without detectable signals in the lung. [I.J. Hildebrandt, M. Iyer, E. Wagner, S.S. Gambhir, Optical imaging of transferrin-targeted PEI/DNA complexes in living subjects, Gene Ther. 10 (2003) 758–764, Copyright (2004) Nature Publishing Group http://www.nature.com/gt/index.html].

signal, D-luciferin has been i.p. injected. The highest levels of transgene expression were observed in tumor tissue and the site of injection. In contrast to ex vivo evaluations, the transfection could be monitored over longer time periods in a single animal.

6.3.3. Growth factors

The epithelial growth factor [EGF] receptor is highly over-expressed in a large variety of cancer cells [194] and might therefore serve as useful target for the delivery of therapeutic genes into tumor. EGF, a 53-residue polypeptide, binds to its receptor, triggering the clustering into coated pits and cell internalization. It has been shown that the tagging of EGF to bPEI25/DNA polyplexes can enhance the cellular uptake into tumor cell lines, due to receptor-mediated internalization, yielding similar transfection efficiency compared to unmodified bPEI–DNA complexes even at lower DNA doses. [60,191]. EGF was incorporated into post-PEGylated polyplexes, either directly conjugated to bPEI25 or, in the final synthesis step, to the distal ends of the PEG spacer arms. Both strategies lead to a 10–100-fold higher transfection efficiency, compared to unmodified complexes, suggesting that PEG interferes only slightly with the EGF receptor binding. To avoid the exposure of polyplexes to further chemical modifications or cleaning procedures, blends of lPEI22, bPEI25–PEG and EGF–PEG–bPEI25 have been used for DNA compaction [133] (pre-PEGylation). The transfection efficiency of this system was comparable to that of those prepared by post-PEGylation, but allowed for a defined particle composition. The pre-PEGylated EGF-labeled PEI/DNA polyplexes have been systemically injected into mice bearing a variety of subcutaneously growing human tumor xenografts. Even though the efficacy of transgene delivery differs between the individual tumors, gene delivery was successfully directed into the tumor tissue [192].

6.3.4. Membrane folate-binding protein

Quickly dividing cells like cancer cells highly over-express the membrane folate binding protein, up-regulating their folate internalization to enable an increased DNA synthesis. Folate has been tagged to several drug or gene delivery systems, all in an attempt to target cancer tissue [195]. A rather simple approach added folic acid to the medium during transfection, which inhibited non-specific interactions with serum components, but failed to mediate cell internalization via the folate receptor [196].

PEGylated polyplexes were tagged with folate to enhance gene delivery to malignant tissues via receptor-mediated endocytosis. Folate–PEG–bPEI [197] or bis-folate–PEG–bPEI [198] conjugates were prepared and used for DNA compaction. Derivatives of moderate substitution degrees have been shown to retain their DNA condensing capacity and endosomolytic activity. When neutrally charged pegylated complexes were used, the enhancement of in vitro transfection efficiency in different cell lines, as compared to either unmodified [197,198] or folate–bPEI-polyplexes [197], was attributed to the receptor-mediated endocytosis. At higher *N*/*P* ratios, adsorptive endocytosis competed with receptor-mediated internalization, reaching the highest gene expression levels using unmodified bPEI–DNA polyplexes.

6.3.5. Integrins

The integrin family includes the heterodimeric transmembrane receptors involved in cell–cell and cell-extracellular matrix interactions, which are amplified in tumor endothelia [199,200]. Several integrins recognize multiple Arg-Gly-Asp (RGD) peptide sequences presented by cell adhesion, serum or extracellular matrix proteins [201], mediate the internalization of matrix compounds [202] or are exploited for the cell entry of viruses like the adenovirus [203]. RGD peptides, like RGDC [156] and CYGGRGDTP [204], have been conjugated to bPEI25 and shown to enhance gene delivery significantly, compared to unmodified bPEI/DNA polyplexes, via ligand–receptor interaction. The selectivity and gene transfer efficiency largely depends on the cell line and appears to be a function of the RGD peptide grafting density, increasing with the number of RGD residues presented in the complex. To combine a charge shielding effect with cell targeting, RGD peptides, such as RGDC [156] or ACRGDMFGCA [157] were attached to the distal end of the PEG chain, grafted to bPEI25 and polyplexes were formed by condensing DNA with the conjugates. While complexes decorated with the ACRGDMFGCA peptide exhibited enhanced transfection

efficiency above that of unmodified bPEI/DNA polyplexes, RGDC-tagged vectors delivered DNA less efficiently than particles lacking the targeting moiety. Whether or not PEG interferes with receptor binding or may be of benefit for integrin-receptor targeting, is still controversially discussed [156].

6.3.6. Antibodies and antibody fragments

Unlike other targeting molecules, which interact with receptors expressed on the surface of a variety of cells, antibodies feature a highly selective binding to their target structure, which is exclusively presented by specific cells. The anti-CD3 antibody, a ligand for the T cell receptor-associated CD3 molecule, was conjugated to bPEI800 prior to DNA complexation. The antibody tagged polyplexes were capable of transfecting a high percentage of human T cell leukemia cells [81] or human primary lymphocytes [205] in a specific manner. In the latter, gene delivery could further be augmented by stimulation with the transcriptional activator phorbol-12-myristate 13-acetate up to 60%.

Unfortunately, potential immunogenicity prevents the often required repeated administration and the complex structure of the antibody enlarges the polyplex size and weakens complex stability. Here, the use of antibody fragments seems favorable, due to their comparably small size and reduced immunogenic potential.

The OV-TL16 antibody binds specifically to the OA3 antigen presented on OVCAR-3 human ovarian carcinoma cells. To direct gene delivery into OVCAR-3 positive cells, the OV-TL16 antibody fragment was linked to bPEI residues of a random PEG–bPEI25 copolymer. The reporter gene expression levels rose up to 80-fold above that have been achieved by unmodified bPEG–PEI/DNA and bPEI/DNA polyplexes, at least partially due to ligand–receptor binding [206].

7. Summary and outlook

In recent years, understanding of the mechanisms behind non-viral gene delivery has expanded and with it the spectrum of methods and materials available. PEI is still considered to be a gold standard compared to other polymers, as it facilitates effective DNA binding and protection, combined with a high endosomolytic competence, which all contribute to the superior transfection efficacy of the corresponding non-viral vectors. The presence of amino groups facilitates the conjugation of targeting moieties and the attachment of charge shielding agents either to PEI itself or to the surface of prefabricated polyplexes. In comparing transfection experiments in vitro and in vivo, lPEI/pDNA polyplexes seem to have an advantage over bPEI-derived vectors, as they facilitate the translocation of exogenous DNA to the nucleus nearly independent from the cell cycle and exhibit a drastically increased cell viability and transfection efficacy.

The improvement of gene delivery by the incorporation of endosomolytic agents or NLS, however, was less than expected for pDNA/PEI polyplexes, inspiring the search for more potent molecules, and a polyplex design, orientated on the different physical properties of linear and branched PEI/DNA complexes. One very straightforward approach appears to be the use of protein transduction domains, as they mediate both, polyplex internalization independent of the endocytotic pathway and translocation of their cargo into the cell nucleus.

It has already been shown that advanced PEI/DNA polyplexes are able to deliver genes into, for example, the lung or tumor tissue successfully by local or systemic application. The first attempts at developing programmable vectors appear to have advantages over other strategies. Using the multivalent but reversible attachment of the shielding component, prevents non-specific interactions at the systemic level efficiently and than, after its intracellular removal, promotes endosomolysis as well as DNA release and transcription. Further improvements may conceivable lead to convenient novel non-viral vectors, combining high efficacy, innocuousness and high stability to be widely applicable for therapeutic gene therapy.

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