Review

Poly(ethyleneimine) and its role in gene delivery

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Abstract

Since the first published examination of poly(ethyleneimine) (PEI) as a gene delivery vehicle, there has been a flurry of research aimed at this polycation and its role in gene therapy. Here we will briefly review PEI chemistry and the characterization of PEI/DNA complexes used for gene delivery. Additionally, we will note various PEI transfection considerations and examine findings involving other polycationic gene delivery vehicles used with cellular targeting ligands. The current state of our knowledge regarding the mechanism of PEI/DNA transfection will also be discussed. Finally, we will survey toxicity issues related to PEI transfection. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The popular press has taken numerous opportunities to herald the promise of gene therapy as a potential cure for several genetic diseases. Although this news might seem fresh and innovative, the transfer of genetic material into living cells has been around for decades. Even in 1966, transfection techniques for mammalian cells were already touted as part of the future of medicine [1]. However, after over 30 years of research, the field of gene therapy has yet to provide an acceptable treatment as a cure for a human disease.

This is not to say that gene therapy has made no progress in the last three decades. Both viral and nonviral gene delivery systems have been used in clinical trials to treat maladies such as cystic fibrosis [2–7] and several forms of cancer [8–11]. With each successive trial the prospect of successful gene therapy treatment for human disease has become more feasible. However, the majority of gene delivery methods have involved primarily adenoviral or liposomal vectors. There exist numerous other possible vectors that are currently under intense scientific investigation. One such vector, poly(ethyleneimine) (PEI), has recently appeared as a possible alternative to viral and liposomal routes of gene delivery.
2. PEI chemistry

PEI is a polymer that has been used for years in common processes such as paper production, shampoo manufacturing, and water purification. The polymer comes in two forms: linear and branched. The branched form is produced by cationic polymerization from aziridine monomers (Fig. 1) via a chain-growth mechanism, with branch sites arising from specific interactions between two growing polymer molecules. Polymer growth is terminated by “back biting,” or intramolecular macrocyclic ring formation. The linear form of PEI also arises from cationic polymerization, but from a 2-substituted 2-oxazoline monomer (Fig. 1) instead. The product (for example linear poly(N-formylethylaminine)) is then hydrolyzed to yield linear PEI. The linear form of PEI is also attainable from the same process as that used to attain branched PEI, but the reaction must take place at relatively low temperature. This method will produce linear PEI molecules with higher molecular weights (up to 25 000 Da), with the linear PEI separating from the branched PEI molecules via precipitation. (These methods are described and reviewed in Ref. [12].) The branched form of PEI has yielded significantly greater success in terms of cell transfection, and is therefore the standard form of PEI that is used for gene delivery. Unless otherwise noted, all references to PEI ascribe to the branched form of the molecule.

The basic unit of PEI has a backbone of two carbons followed by one nitrogen atom; as already mentioned, the complete polymer can be linear or branched (Fig. 1). The branched form of PEI contains 1°, 2°, and 3° amines, each with the potential to be protonated. This gives PEI the attribute of serving as an effective buffer through a wide pH range. With nitrogens appearing as one out of every three atoms

![2-Substituted 2-Oxazoline](image1)

![Aziridine](image2)

![Linear PEI*](image3)

![Branched PEI](image4)

Fig. 1. Structures of PEI precursors and end products. *Aziridine can also yield linear PEI under certain conditions.
in the PEI backbone, any benefits of branching and protonability quickly accumulate in relation to the overall polymer size.

Several suppliers offer PEI in a variety of reported molecular weights. However, the actual molecular weight of a given PEI product appears to be reported differently depending upon the particular company supplying the chemical. There are three PEI products that predominate in the transfection literature— one available from Fluka (Milwaukee, WI, USA; Cat. No. 03880) with a reported molecular weight of 600 000–1 000 000, one from Polysciences (Warrington, PA, USA; Cat. No. 00618) with a reported molecular weight of 70 000, and one from Sigma (St. Louis, MO, USA; Cat. No. 40 872-7) with a reported weight average molecular weight of 25 000. Fig. 2 shows the results of gel fractionation chromatography performed on the three products (against seven polyethylene glycol (PEG) standards in 0.5 M NaCl, using a Hydroapore 87-S03-C5 size exclusion column (Varian, Walnut Creek, CA, USA)). The molecular weights reported in the figure vary from those reported by suppliers because of differences in the analytical methods and/or standards used.

The protonability of PEI has already been mentioned as important to PEI’s success as a gene delivery vehicle. A useful descriptor of PEI’s protonability is its pKₐ, which has remained elusive because rigorous analysis of experimental data to obtain PEI’s pKₐ would require one to include an ionization constant for every amine group [13]. The number of amine groups can easily exceed 1000, depending on the molecular weight of the PEI being analyzed. Suh et al. reported on the percentage of

Fig. 2. Gel fractionation chromatograms of three PEI products used for gene delivery, measured against PEG standards. (NMW = nominal molecular weight.) (1) Polysciences NMW 70 000; (2) Sigma NMW 25 000; (3) Fluka NMW 600 000–1 000 000. The number average molecular weights, Mₙ, weight average molecular weights, Mₐ, and polydispersity indexes, P.I., for the three PEIs as determined by gel fractionation chromatography [25] are as follows:

<table>
<thead>
<tr>
<th></th>
<th>Polysciences</th>
<th>Sigma</th>
<th>Fluka</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mₙ</td>
<td>8.400±2 800</td>
<td>700±200</td>
<td>75 800±12 700</td>
</tr>
<tr>
<td>Mₐ</td>
<td>133 800±10 800</td>
<td>8000±2800</td>
<td>155 500±4 500</td>
</tr>
<tr>
<td>P.I.</td>
<td>16.99±5.20</td>
<td>11.66±4.82</td>
<td>2.08±0.31</td>
</tr>
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unprotonated nitrogens at various pHs for two concentrations of one molecular weight of PEI [13]. The data reported imply that the amount of PEI protonation depends, in part, on its concentration. However, the concentrations of PEI that are used for transfection were not examined in the paper, so the protonation values reported do not accurately reflect what occurs in transfecting PEI, and they should not be used for such a purpose. With each PEI nitrogen having its own local environment influencing its protonability, perhaps what is more important than obtaining a particular PEI molecule’s pKₐ is its overall buffering capacity over a range of pHs.

3. Characterization of PEI/DNA complexes

In the presence of sufficient PEI, DNA will condense [14]. The extent of condensation depends on the polymer:DNA ratio [15]. PEIs with branched structure condense to a greater extent than do linear PEIs [14]. The induction of DNA condensation, in addition to providing compact colloids for endocytosis, might also afford some protection to the carried DNA from nuclease digestion as has been shown for PEG–poly(l-lysine)/DNA complexes [16].

The shape of polycation-condensed DNA has been examined in great detail, with condensate shapes taking on a variety of forms. A given plasmid/polycation combination can condense into more than one configuration, as shown with T7 DNA condensing into both toroids and tubular micelles (rods) in the presence of polylysine [17]. However, differences in specimen preparations can alter the morphology of condensed complexes [18], and it has been suggested that rod conformations are actually artifacts induced by sample preparations [19]. Many laboratories have examined the effects of different polycationic condensing agents on DNA conformation, and toroidal condensates have predominated [19–23]. This suggests that the ultimate shape of polycation/DNA complexes might not depend on the specific polycation used, but might be due to a more general factor such as the kinetics behind polycation-induced DNA condensation. Kinetics have been pointed out by Dunlap et al. as a possible explanation for the rounded, globular forms taken on by DNA in the presence of linear or branched PEI [14]. This group also noted that in the presence of either form of PEI, plasmid DNA condensed into bundled, folded loops as opposed to winding.

The sizes of PEI/DNA complexes have been found to be within the range 20–40 nm using atomic force microscopy [14]. The sizes were fairly consistent between complexes made with linear or branched PEI, although the branched PEI also yielded a small amount of much larger condensates (over 240 nm in one case). Using electron microscopy, Tang and Szoka found toroidal PEI/DNA structures of 55±12 nm [23]. The group also examined complexes in solution using dynamic light scattering and found apparent diameters ranging from 90 to 130 nm. The number of plasmids within a PEI/DNA complex remains unknown as of yet, but lipopolyamine/DNA complexes have been noted as containing approximately 100 plasmids [24].

The surface charge of PEI and PEI/DNA complexes has been examined in terms of ζ potential [25]. It was found that while a certain sample of branched PEI had a ζ potential of 37 mV in solution, this value was lowered significantly to 31.5 mV when the PEI was allowed to complex with DNA (at a 7.5:1 PEI nitrogen to DNA phosphate ratio). Centrifugation of PEI/DNA complexes was seen to lower the ζ potential further, this time to 29.2 mV. The surface charge of the transfecting colloids is thought to be an important factor in the complexes’ association with plasma membranes.

4. Transfection considerations

DNA transfection using polycations such as PEI, as with adenoviral transfection, is transient because of the lack of integration into the host genome, as well as a lack of (episomal) replication [26]. However, unlike adenoviral transfection methods, PEI-mediated gene delivery has the potential to transfet cells with larger pieces of genetic material. (Reviewed by Abdallah et al. [27].) Upper limits to the size of virally-delivered plasmids are naturally set by the size of the particular viral head used: an adenovirus holds up to 7.5 kilobases (kb) [28], while an aden-associated virus can carry between 2.5 kb [29] and 4.5 kb [30] of genetic material. Although
retroviral transfection does offer permanent expression of the delivered gene, retroviral transfection requires actively dividing cells [30,31].

The ability of PEI to transfect a wide variety of cells is well established. Boussif et al. have documented PEI-mediated transfection in 25 different cell types, including 18 human cell lines as well as pig and rat primary cells [32]. Gene delivery experiments have been performed in vivo using newborn [33] and adult [34] mice as well as on Sprague-Dawley rats [35]. Abdallah et al. reported no morbidity in the mice used over their 3 month experiment course [34].

The ratio of PEI nitrogens to DNA phosphates is important in terms of transfection efficiency and cell toxicity. Polymer/DNA complexes with an overall positive charge can activate complement, and reducing the +/− charge ratio of the complexes reduces complement activation as well as the amount of cell death associated with transfection [36]. Ferrari et al. hypothesized that circulating proteins can bind to and inactivate cationic polymer/DNA complexes [37], and Boussif et al. have found that transfection efficiency is increased when serum is present by using a more-concentrated PEI/DNA solution for transfection, lending support to the hypothesis of circulating proteins binding to polycation/DNA complexes [32]. The binding of circulating proteins could also induce recognition by cells of the reticuloendothelial system, which would halt the transfection process in vivo.

One way to minimize the effects of protein binding to PEI/DNA is through manipulation of the PEI amine to DNA phosphate ratio. Ratios ranging from 5:1 to 13:5:1 have all been used successfully in the literature, with each proportion having its own merits. Boussif et al. tested branched-PEI transfection with charge ratios ranging from 4.5:1 to 135:1 and reported maximal transfection efficiency in vitro within the 9–13.5 PEI nitrogens per DNA phosphate range [33], while Ferrari et al. used linear PEI at a 5:1 ratio in an attempt to transfect cells with neutrally charged complexes [37].

Another way to minimize the binding of circulating proteins to polycationic transfection complexes is through surface modification. Several types of nanoparticles have undergone such alterations in the past in an attempt to circumvent uptake by phagocytic cells. Examples of modified nanoparticles include polystyrene, poly(methyl methacrylate), poly(β-hydroxybutyrate), and poly(lactic acid), with surface modifiers including various poloxamers, ethoxylated glycerols, and PEG [38]. It has also been shown that PEG-epoxide will readily interact with PEI, and that polystyrene surfaces, after preadsorption of PEI and subsequent coupling with PEG, will act to exclude proteins such as fibrinogen from binding to the surface [39]. The idea that PEG can prevent protein binding has been carried to PEI/DNA complexes, with PEG and PEI being used together to form block copolymers. The PEI in these copolymers is still able to interact with DNA, and the resulting complexes have been described in some detail by Vinogradov et al. [40]. This type of complex modification shows promise for the in vivo use of PEI for gene delivery.

When forming transfection complexes by the addition of PEI to a DNA solution, it is unlikely that the PEI will form a continuous shell around the resulting complexes. These colloids will have areas where smaller molecules will be able to fit. It has been shown that a subsequent addition of smaller PEI molecules to formed PEI/DNA complexes will result in complexes with increased packing of amines about the DNA [25]. The larger number of PEI amines per PEI/DNA complex yielded complexes with higher buffering capacities, which presumably enabled more efficient endolysosomal escape. This, in turn, allowed a greater number of plasmids to reach the nucleus for transcription, as evidenced by increased transfection efficiency.

The pH and molecular weight of PEI used for transfection have also been examined. Although differing pHs of PEI solutions do not make any significant difference in transfection efficiency [33,41], the molecular weight of the PEI used for transfection does. Abdallah et al. examined the transfection efficiencies of three molecular weights of PEI in vivo and found that PEI with a reported molecular weight of 25 kDa yields higher transfection efficiency than PEIs of higher reported molecular weights [34]. Godbey et al. also examined the effect of PEI molecular weight on transfection efficiency using PEIs with lower molecular weights in vitro. They determined that as molecular weight was increased, so was transfection efficiency (Fig. 3)
This result for low molecular weight PEIs is consistent with the finding that interpolymer systems with relatively few salt bonds will dissociate during dilution [42], which implies that PEI/DNA complexes made with smaller PEI molecules will dissociate more easily and provide lower transfection yields. However, the largest (and highest-transfecting) molecular weight of PEI tested by Godbey et al. had a similar weight average molecular weight to that of the largest (and lowest-transfecting) PEI examined by Abdallah et al. described above. To resolve the apparent conflict, the two PEI samples (from Polysciences and Fluka) have since been tested directly, with the Polysciences product yielding a significantly higher transfection efficiency [25]. This difference is most likely due to the differences in polydispersity between the two products. (Gel fractionation chromatography and polydispersity analyses of the two products are given in Fig. 2.)

Another consideration implied by the molecular weight verses transfection efficiency data is that it is the size of the resulting complexes that affects transfection efficiency. Ogris et. al. have found that smaller (40 nm) transferrin–PEI/DNA complexes, made at low salt concentrations, have lower transfection efficiencies than larger (>1000 nm) complexes made from the same components at physiological salt levels [43]. Perhaps the smaller complexes have decreased cellular uptake or increased degradability levels. These are possibilities that are currently under investigation.

5. Targeting

PEI has been coupled with different ligands for the purpose of cell targeting. Examples of ligands used include RGD peptide sequences [44], antiCD3 [45], and galactose for hepatocyte targeting [46]. Additionally, because poly(L-lysine) (PLL) and PEI are both polycationic polyamines, any successful targeting methods that have been used for PLL/DNA complexes ought to apply to PEI/DNA complexes as well. One example of a targeting ligand that has been used with both polymers is transferrin, which has been attached to both PLL/DNA and PEI/DNA complexes for the purpose of targeting transfection to specific cell types [20,43,45,47]. Other ligands attached to PLL include antiCD4 [48], recombinant gp120 (the envelope protein of HIV) [48], folate
[49], and synthetic peptides (attached with an integrin binding segment) [50]. Even low density lipoprotein has been used as a ligand for cell targeting through attachment to stearyl (hydrophobized) PLL molecules [51]. This form of cell targeting is often responsible for improved uptake of transfecting complexes into cells, and will result in higher transfection efficiencies (provided the cell recognizes the targeting ligand used) [45].

While the attachment of ligands to transfecting complexes to achieve targeted endocytosis is one form of cell targeting, another method entails the use of cell-specific promoters to achieve targeted expression of delivered DNA. Using this sort of promoter targeting, many different cell types will endocytose the transfecting complexes but only those cells that recognize the delivered plasmids’ promoter will transcribe and express the delivered gene. Cowan et al. used this method to target vascular endothelia by using the promoter for the intercellular adhesion molecule 2 gene [52]. Several different cell-specific promoters have been identified thus far, and each serves as a potential genetic marker for targeted gene delivery.

6. Mechanism

The mechanism by which PEI transfects cells begins with entry of PEI/DNA via endocytosis (Fig. 4A,B). In experiments involving fluorescently labeled PEI/DNA complexes, it has been shown that the complexes initially form aggregates on plasma membrane surfaces [53]. Experiments that used lipopolyamine/DNA particles for transfection revealed that such complexes bound to membrane components involved in Ca\(^{2+}\)-mediated cell anchoring to the extracellular matrix. Only adherent cells were involved with uptake of the complexes [24]. In examining CaPO\(_4\) -mediated transfection, Coonrod et al. used vinblastulin to depolymerize microtubules and thus indicate entry of complexes into cells via endocytosis [54]. The same group also used cytochalasin B to interrupt microfilament formation and keep transfection complexes in the outer peripheries of cells after endocytosis, thus blocking DNA transport at endosome/lysosome fusion [54].

Following endocytosis, normal cellular trafficking usually has endocytosed particles being directed to lysosomes for degradation. Improved transfection efficiency by the use of chloroquine, which represses lysosomal degradation, would imply endocytosed transfection complexes do eventually meet up with lysosomes for destruction. This has been shown to be the case with both PLL [49] and PEI [43]. Using a different approach, Chiu et al. demonstrated lysosomal degradability of PLL (as well as other \(\alpha\)-amino acids) through detection of lower molecular weight fragments in cytoplasms after a time [55]. These results do not inherently translate to other gene delivery vehicles though, because the group did not see any degradation of poly(\(\alpha\)-lysine) in the same experiments. It is generally assumed, however, that PEI/DNA complexes are trafficked to lysosomes for degradation after endocytosis.

Lysosomal membranes contain V-ATPases, which pump H\(^+\) at the expense of ATP [56]. A charge gradient would theoretically build because of protons being pumped into the lysosomes, but there is also an influx of Cl\(^-\) which relieves this gradient [56]. It has been hypothesized that the increased Cl\(^-\) concentration, in turn, increases lysosomal osmolarity, with water rushing in to relieve the gradient resulting in lysosomal swelling and bursting [33,57]. Even though lysosomes eventually burst, they are still quite successful at degrading endocytosed particles beforehand. Transgene delivery is often interrupted within endolysosomes because of nucleases that reside within lysosomes. PEI is thought to be a good gene delivery vehicle because of its ability to accept the protons pumped into endolysosomes. By accepting protons, PEI could raise endolysosomal pH, which would alter protein folding within the endolysosome and perhaps inactivate degradative enzymes. The enzymes might be inactivated long enough for endolysosomal bursting to occur before plasmid digestion has taken place. As discussed earlier, modified PEI/DNA particles with increased buffering capacities have been shown to increase transfection efficiency, which lends support to the above hypothesis.

After endolysosomal disruption, the delivered plasmids eventually reach the nucleus for transcription. PEI/DNA complexes that were microinjected into cell cytoplasms were translocated into the nuclei of these cells, without dependence on membrane
rupture or cell mitosis [58]. The same result was also seen in experiments using exogenously-administered PEI/DNA particles that went through normal endocytic trafficking, with complexes entering nuclei in the form of large, discrete structures (Fig. 4C) [53]. The fact that complexes made it into cell nuclei intact shows that it is not necessary for PEI and DNA to separate prior to nuclear entry of the delivered plasmids. The path of PEI/DNA complexes from endolysosome disruption to nuclear entry has not yet been fully explained, but it is doubtful that it occurs by mere diffusion. Cytoplasmic diffusion has been projected as slow because of the cytoplasm's relatively-high viscosity [59]. Vesicle transport may depend on the cytoskeleton [60–63], and with endocytosed PEI/DNA complexes entering nuclei in what appear to be vesicles it might follow that the complexes also travel along cytoskeletal tracks.

The mechanism by which PEI/DNA complexes cross the nuclear membrane can only be speculated on at this time, but it may involve the coating of complexes with a lipophilic layer. Whether this
coating comes from anionic phospholipids adhering to the cationic exterior of the complexes, or the coating is actually made from fragments of the membrane of burst endolysosomes, it is possible that PEI/DNA complexes enter cell nuclei as the result of the coating's fusion with the nuclear membrane. Cationic liposomes have been shown to interact with anionic lipids [64], and to fuse with endosomal membranes to thereby release their carried DNA [65]. Phospholipid-coated PEI/DNA complexes might be released into cell nuclei in the same way. During transfection is work performed by Lambert et al. regarding neuronal cell transfection, which demonstrated no excitation of neuronal cells caused by the transfection process (although PEI concentrations above 150 μM were toxic to neuronal cells) [70]. These data collectively suggest that low concentrations of PEI will not harm plasma membranes. However, endosomal concentrations of PEI might be elevated to a point where permeabilizing effects might occur, although this has yet to be proven.

8. Conclusions

Although it has been used in a variety of chemical processes for many years, PEI has recently been used successfully for transfection both in vitro and in vivo. As a polycation, PEI will spontaneously adhere to and condense DNA to form toroidal complexes that are readily endocytosed by cells. The presence of multiple unprotonated amines in the complexes is thought to buffer endolysosomal pH, thus allowing cytoplasmic release of the PEI/DNA before lysosomal degradation can occur. The PEI/DNA complexes are eventually translocated into cell nuclei, but it remains to be seen what effects this has on host cell transcription.

Numerous physical characterizations have been performed on PEI/DNA complexes, including size, shape, surface charge concentration, and buffering capacity analyses. These characterizations describe properties that are important to the success of PEI transfection, and may be useful for other nonviral transfection methods as well (just as poly(L-lysine) targeting research has been applied to PEI-mediated gene delivery). Future nonviral vectors could also be designed based on data obtained for PEI mediated transfection. Increasing transfection efficiency while reducing toxicity must be accomplished before PEI can ultimately be used for efficacious gene therapies, although progress toward this end continues to be rapid.

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References


