Dendritic Polyamines: Simple Access to New Materials with Defined Treelike Structures for Application in Nonviral Gene Delivery

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Dedicated to Professor Dr. Fritz Vögtle on the occasion of his 65th birthday

Polycationic dendrimers are interesting nonviral vectors for in vitro DNA delivery. We describe a simple approach to the synthesis of dendritic polyamines with different molecular weights and adjustable flexibility (degrees of branching; DB). Both parameters influence the transfection efficiency and the cell toxicity of the polymer. Functionalization of hyperbranched polyethylenimine (PEI) by a two-step procedure generated fully branched pseudo-dendrimers (analogues of polypropylenimine (PPI) and polyamidoamine (PAMAM) dendrimers). The DNA transfection efficiencies observed for these polymers depended on the cell line investigated. The highest efficiencies were observed for polymers whose unfunctionalized PEI cores had molecular weights in the range $M_w = 6000–25\,000\ g\,mol^{-1}$. The cytotoxicity of the dendrimers generally rises with increasing core size. The data collected for NIH/3T3 and COS-7 cells indicate a maximum transfection efficiency at around 60% branching for the PPI analogues, and at a PEI-core molecular weight of $M_w = 25\,000\ g\,mol^{-1}$. PAMAM functionalization of PEI ($M_w = 5000$ and $21\,000\ g\,mol^{-1}$) leads to polymers with little or no cytotoxity in the cell lines investigated.

Introduction

The most concrete structural evidence currently available for use in the rational design of nonviral poplycationic gene delivery systems is the X-ray structure of a DNA–histone complex.[1] As a result of the problems associated with viral gene transfection, such as immune response and limited selectivity, the search for nonviral alternatives remains an important challenge.[2] However, nonviral polycationic transporters still lack three orders of magnitude in transfection efficiency as compared to viral vectors. In the past decade, several (more or less systematic) approaches have been taken to this problem, such as the use of cationic amphiphiles, polymers, or block copolymers.[3–8] Another approach is to use perfect polyamine dendrimers[3,6,9] to mimic the globular shape of the natural DNA–histone complex. However, the synthetic effort required to obtain dendritic structures in the size-range of the natural protein complex (around 8 nm) is tremendous (12–18 steps)[1] and in some cases a partially destroyed (hydrolyzed) dendritic backbone shows even higher transfection efficiencies than the intact dendrimer.[6,7] More recently, self-assembled amphiphilic dendrons have been prepared[10] that mimic the natural histone complex in size, surface charge, and flexibility and achieve good DNA complexation and high transfection efficiencies. A simple access route to dendritic nanoparticles of different sizes and flexibilities (degree of branching) would allow molecular structure to be correlated with transfection efficiency.

We describe herein an efficient two-step approach to the synthesis of dendritic polyamines from hyperbranched polyethylenimine (PEI). Some of the reported hyperbranched PEIs ($M_w = 5000$, 25 000, and $M_r = 600\,000\ g\,mol^{-1}$) are commercially available, all others were prepared by BASF AG. In only two synthetic steps, fully branched analogues of polypropylenimine (PPI, Astramol®, DSM Fine Chemicals) and polyamidoamine (PAMAM, Starburst®, Dendritic Nanotechnologies) dendrimers can be prepared that have high molecular weights and a narrow molecular weight distribution. Our strategy also allows the degree of branching (DB) to be controlled in the case of PPI analogues. The degree of branching has a significant effect on the flexibility of these macromolecules and hence their ability to complex and transport DNA. The synthesized polymers were tested for in vitro transfection efficiency and cell toxicity and these properties were correlated to the degree of branching and molecular weight.

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Results and Discussion

Hyperbranched PEI (**1**) is a readily available material that was initially developed as an additive for use in paper production and has since found many other applications, for example in complexation agents, surface coatings, etc. A major drawback of large-scale technical products like PEI for many high-end applications is the relatively broad molecular weight distribution (MWD) and low DB of some such products. We prepared several well-defined hyperbranched PEIs either by using controlled reaction conditions and performing the synthesis on a smaller scale, or by membrane filtration of the technical products (Scheme 1, Figure 1).[11] Both methods gave PEIs with narrow molecular weight distributions (1.2–1.7) and degrees of branching ranging from 65 to 75% (84% for the PEI with M_w = 800 g mol^{-1}), as determined by inverse-gated ^{13}C NMR spectroscopy (see the Supporting Information).[12] The highly defined structures of these globular molecules allow them to serve as branching scaffolds for more elaborate dendritic architectures.

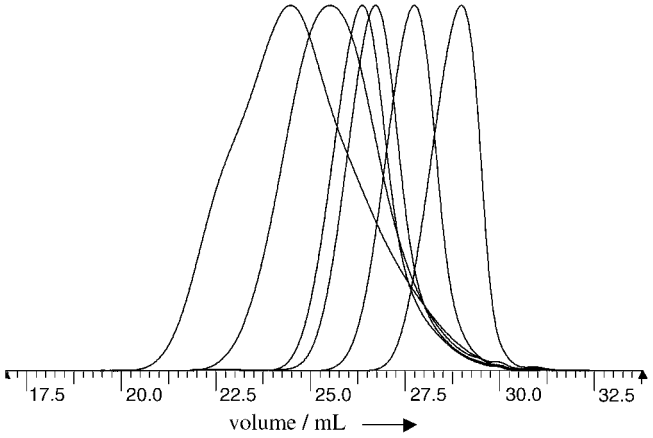
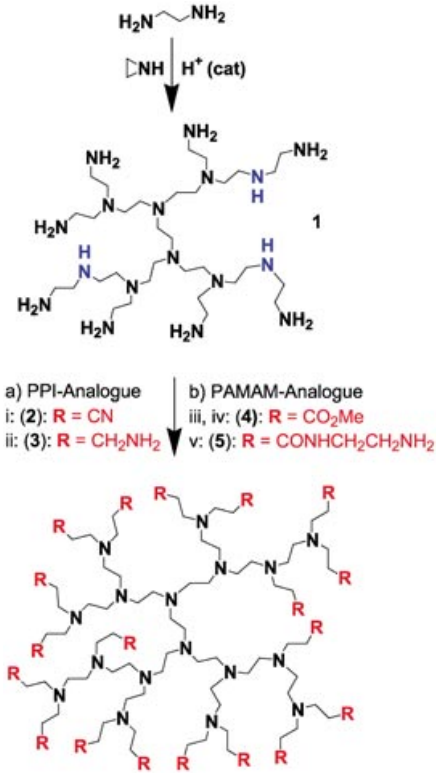


Figure 1. *Gel permeation chromatograph of some PEIs used in our experiments. From right to left: $PEI_{0.8}$ (M_w = 800 g mol^{-1}, MWD = 1.3, DB = 84%), PEI_2 (M_w = 2000 g mol^{-1}, MWD = 1.2, DB = 73%), PEI_5 (M_w = 5000 g mol^{-1}, MWD = 1.4, DB = 72%), PEI_6 (M_w = 6000 g mol^{-1}, MWD = 1.4, DB = 70%), PEI_{21} (M_w = 21 000 g mol^{-1}, MWD = 2.0, DB = 62%), PEI_{25} (M_w = 25 000 g mol^{-1}, MWD = 2.6 [1.7 after dialysis], DB = 65%). DBs were determined by inverse-gated ^{13}C NMR spectroscopy.*

Dendritic PPI analogues

We recently developed a general method for converting hyperbranched polyglycerols into fully branched dendritic polymers by applying one dendrimer sequence.[13] This method can also be used to prepare close analogues of the PPI and the PAMAM dendrimers (Scheme 1). This simple process allows the generation of high-molecular-weight dendritic polymers in only two synthetic steps, while the corresponding "perfect" structures have to be prepared by a multistep process.[14,15] Dendritic PPI analogues were prepared by conversion of a PEI (M_w = 25 000 g mol^{-1}, MWD = 1.7) into a dendritic polyamine ($M_w \approx$ 60 000 g mol^{-1}) in only two steps (Scheme 1). This product corresponds to an eighth-generation perfect PPI dendrimer (16 synthetic steps!) with a molecular weight of M_w = 60 276 g mol^{-1}. However, like our analogues, higher generations of perfect dendrimers prepared by using a divergent approach are not monodisperse.[16]



Scheme 1. *Functionalization of PEI to obtain a) PEI/PPI polymer **3**: i) CH_2=CHCN in water, 25 °C, 2 days; ii) $LiAlH_4/AlCl_3$ in THF, 25 °C, 1 day; or b) PEI/PAMAM polymer **5**; iii) CH_2=CHCOOMe, THF, 25 °C, 4 days; iv) CH_2=CHCOOMe, 25 °C, 8 days; v) $H_2NCH_2CH_2NH_2$, 50 °C, 8 days. THF, tetrahydrofuran.*

The protocol we used for the conversion of hyperbranched PEI into a dendritic PPI analogue was initially developed by Vögtle et al.[17] and later optimized by Meijer[15] and Mülhaupt.[18] Michael addition of acrylonitrile to hyperbranched PEI (**1**) occurs almost quantitatively and the dendritic nitriles **2** were obtained in high yields (> 95%). This process can be monitored easily by IR spectroscopy; the disappearance of the NH band and the appearance of a CN band in the spectrum indicate the progress of the reaction (Figure 2a). Another interesting feature of this process is that it provides control of the degree of branching. Several different DBs ranging from 50 to almost 100% were obtained by adding varying amounts of acrylonitrile to the PEI solution (Figure 2b). There is a linear correlation between the DBs (determined by inverse-gated ^{13}C NMR spectroscopy) and the ratios of acrylonitrile/NH in the reaction mixture. Our approach thus allows precise DB adjustment and control of the polymer flexibility.

Several reduction methods were investigated for the conversion of the polynitrile into a polyamine in the second step of the preparation of dendritic PPI analogues. While catalytic reduction (Raney Ni or Co with H_2 in methanol) did not go to completion or resulted in hydrolysis of the dendrimers, treatment of the polynitrile with $LiAlH_4/AlCl_3$ led to the formation of the corresponding dendritic polyamines in good yields (50–

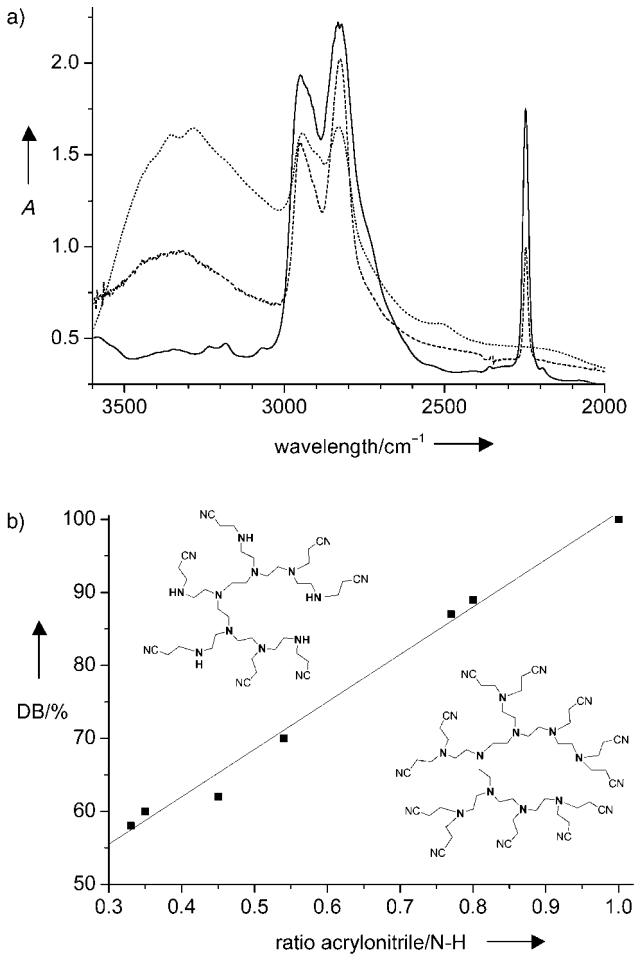


Figure 2. *a) IR spectra of pure PEI_{25} ($M_w = 25\,000\ g\,mol^{-1}$; small dots) and PEI_{25} functionalized by treatment with reaction mixtures with two different acrylonitrile/NH ratios (big dots, 50%; line, 100%). b) Linear correlation between the DB of $PEI_{25}PPI$ (determined by inverse-gated ^{13}C NMR spectroscopy; DB of PEI_{25} substrate, 65%) and the acrylonitrile/NH ratio in the reaction mixture.*

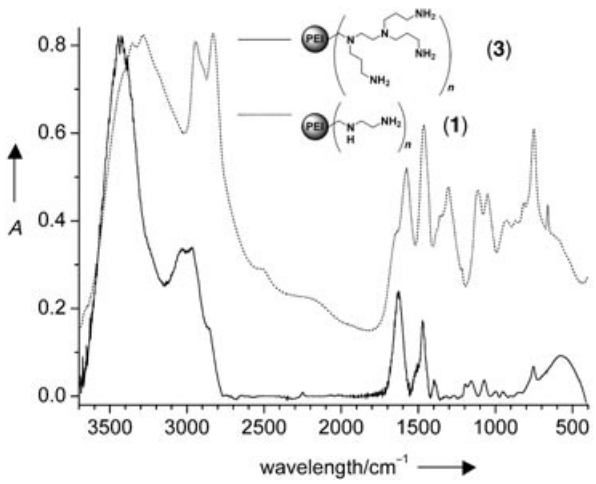


Figure 3. *IR spectra of PEI_{25} (**1**, dots) and the $PEI_{25}PPI_{100}$ pseudodendrimer (**3**, line): $\tilde{\nu} = 3300–3500$ (N–H), 2800–3000 (C–H), 1630 (prim. N–H), 1580 (sec. N–H), 1450–1480 cm^{-1} (C–H).*

70% after dialysis). The IR spectra (Figure 3) of the products indicate that almost complete conversion of the nitrile groups into amine groups was achieved (cf. Figure 2a). The spectra also indicate that the products are structural homogeneous (only dendritic and terminal groups) since only very few bands are present, as expected for a pseudodendritic structure.

Dendritic PAMAM analogues

We used the same concept to prepare dendritic PAMAM analogues with high molecular weights (Scheme 1). Addition of PEI (**1**) to methylacrylate and subsequent treatment of the PEI methylester **4** with ethylene diamine gave rise to the dendritic polyamidoamine **5** in high yield (>95%). As a result of the lower reactivity of methylacrylate compared to acrylonitrile, the highest conversion of amino groups reached was about 93%. The extent of conversion was calculated by integration of the ^1H NMR spectra of the reagents and products. A higher degree of functionalization (almost 100%, no linear units, only terminal units detectable by ^{13}C NMR spectroscopy) could be achieved by using methylacrylate as a solvent in a second step. A drawback of methylacrylate addition, however, is the resulting lack of accessibility of lower DBs. In contrast to acrylonitrile, methylacrylate has to be added to PEI (**1**) in large excess to avoid cross-linkage. In the second reaction step, we also used inverse addition (ester **4** into ethylene diamine) to avoid cross-linkage caused by double amide formation by an ethylene diamine unit. As a result of the rather high reaction temperature (120–140°C) used for the reaction at the beginning of these experiments, partial degradation of the PAMAM shell was observed, as described in the literature.[19] Use of a lower reaction temperature (60°C) leads to incomplete conversion and results in PAMAM analogues **5** with a functionalization of 90% (DB≈95%) after two days. Complete conversion of the ester groups is achieved after seven days at room temperature.

Both dendritic polyamines **3** and **5** are available on a multigram scale after only two synthetic steps. In addition, the molecular weights (1000–60 000 $g\,mol^{-1}$) of both polymers and the degree of branching (50–100%) of PEI/PPI dendrimer **3** can be tailored to provide dendritic nanoparticles (2–15 nm) with different structural flexibilities.

Gene transfection with dendritic polyamines

The influence of the DB (0–23%) of copolymers of ethylene imine and *N*-(2-hydroxyethyl)-ethylene imine on their gene transfection efficiency was recently reported by Kissel et al.[20] The synthesis of low-molecular-weight PEIs with a DB of 50% has also been reported by Kissel et al.[21] The DB was observed to have an influence on gene transfection; more-branched polymers form smaller complexes and are more efficient in gene transfection. However, the polymers examined by Kissel et al. have a molecular weight of about 2000 $g\,mol^{-1}$, which does not correspond to the size of the natural DNA–histone complex.[1] Cheradame et al. recently reported the synthesis of linear copolymers from 2-ethyl-2-oxazoline and ethylenimine

and/or propylethylenimine units.[22] Incorporation of 2-ethyl-2-oxazoline units into the polymers did not affect gene transfection, while polymers with propylenimine units were less efficient than those containing only ethylenimine. The researchers also showed that, in one cell line, linear PEI molecules with a molecular weight of 3.6 kDa have a transfection efficiency similar to that of branched PEI with a molecular weight of 25 kDa. They concluded that the flexibility of the polymer plays an important role in transfection.

Our goal was to prepare dendritic polymers with higher molecular weights and to investigate the structure–activity relationship between the size and flexibility (DB) of these nanoparticles and their transfection efficiency in different adherent cell lines. For this purpose, the above-described dendritic polyamines (Scheme 1) were evaluated in transfection experiments. All polymers were either dissolved in water or first diluted with $H_2PO_4^-/HPO_4^{2-}$ buffer solution (pH 6) then further diluted with water to a final concentration of 1 mg mL^{-1}. The resulting solutions then underwent sterile filtration. The transfection capacity of the dendritic polyamines was determined by using the plasmid pCMVβ as a reporter construct, and the dendrimers were tested in various adherent cell lines. One day before the start of the transfection experiments the cells to be used were plated in complete Dulbecco modified eagle medium (DMEM) in 96-well plates with a cell density of 2×10^4 per well.[7] Each transfection experiment was run with a constant amount of DNA (usually 1 μg per well) in combination with four different amounts of dendritic polyamine (1, 2, 3, and 4 μg per well) to allow the optimal DNA/dendrimer ratio and possible cytotoxic effects of the polymer to be investigated. The plasmid DNA and the polyamines were diluted in 30 μL and 20 μL DMEM medium without serum, respectively. The two solutions were mixed and incubated for approximately 10 minutes to allow complex formation. The complexes were then transferred, together with 150 μL DMEM medium containing serum, onto the cells from which the medium was previously removed. After incubation for approximately 4 hours, the complex-containing medium was removed and fresh complete DMEM was added to the cells. The β-galactosidase activity of the cells was measured 48 h later by a β-Gal assay,[23] and the results were used to determine the transfection efficiencies of the dendrimers. Untransfected and transfected cells were examined microscopically before the β-Gal assay was performed to investigate whether any potentially cytotoxic effects were induced by transfection.

Influence of molecular weight

The dependence of gene transfection efficiency upon molecular weight was initially analyzed with regard to unfunctionalized polyethylenimines. PEIs with different molecular weights (800–600 000 g mol^{-1}, $PEI_{0.8}$–PEI_{600}) were used to transfect four different cell lines (NIH/3T3, CHO-K1, Cos-7, and HeLa). The results of these experiments indicate that two important parameters have to be considered: the molecular weight of the polyethylenimine and its cytotoxicity (Figure 4a, b). The unfunctionalized hyperbranched polyethylenimines all had DBs within a narrow range (65–75%; 84% for $PEI_{0.8}$) according to ^{13}C NMR spectroscopy results.

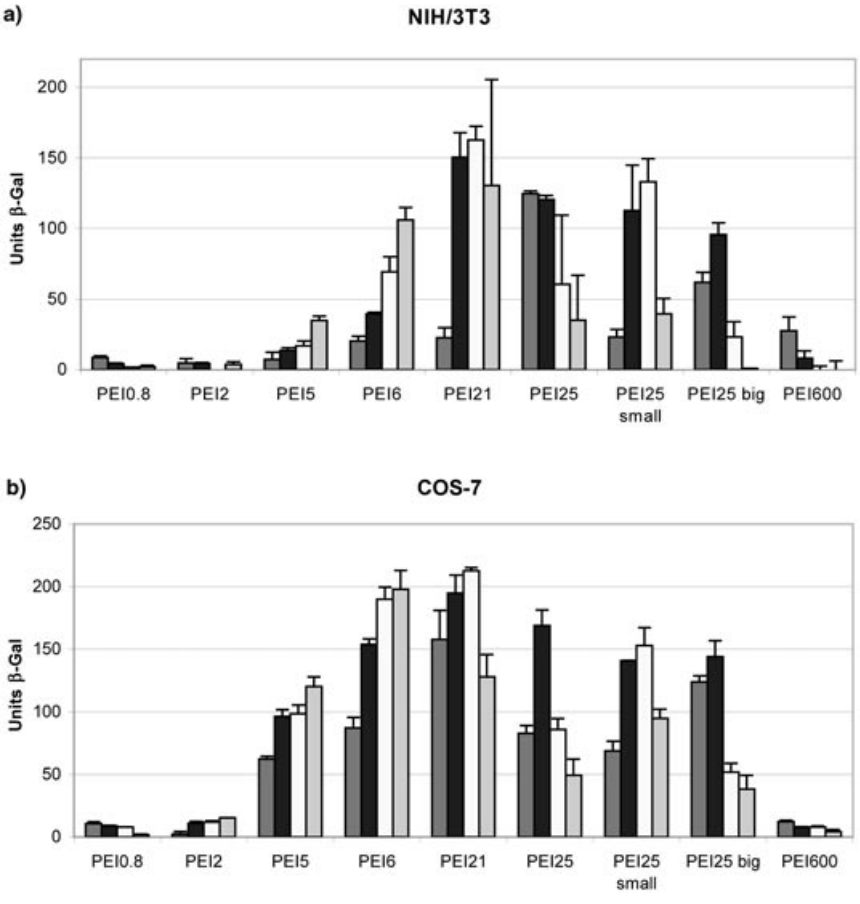


Figure 4. *Transfection efficiencies of several PEIs in a) NIH/3T3 and b) COS-7 cells, expressed as β-Gal units. Increasing amounts of polyamine (1–4 μg (left to right) per well of a 96-well plate) were used for the transfection experiment, in combination with 1 μg DNA. The results shown represent the average of the efficiencies determined for two replicates that have been performed in parallel. PEI_{25} was dialyzed (M_W cut-off, 20 000 Da) to separate the low-molecular-weight fraction (PEI_{25} small) from the high-molecular-weight fraction (PEI_{25} big). 166 β-Gal units were detected in NIH/3T3 cells after transfection with SuperFect®.*

It was reported recently that low-molecular-weight polymers only loosely condense DNA and form big aggregates. This problem can be solved either by attaching the small PEIs to a poly(ethylene glycol) star[24] to obtain polymers with a molecular weight of 19000–26000 g mol^{-1}, or by attaching L-lactic acid/cosuccinic acid oligomers to the PEIs.[8] The modified polymers show higher gene transfection efficiencies than the unmodified polymers with low molecular weights. Our results clearly show that higher-molecular-weight polyethylenimines ($M_w > 5000$ g mol^{-1}) are better-suited to gene transfection. However, depending on the cell line investigated, cytotoxicity may also rise with increasing molecular weight. These two factors determine the optimum molecular weight (M_w) of a PEI for transfection: around 6000 g mol^{-1} (sensitive cell lines) to 21000/25000 g mol^{-1} (relatively robust cell lines). Polymers with molecular weights of 600000 g mol^{-1} or less than 5000 g mol^{-1} showed reduced transfection efficiencies compared to polymers with weights within this optimum range in most cases. This observation supports an analogy between the size of the naturally occurring DNA–histone complex, which has a diameter of 8 nm,[1] and the optimal sizes of the dendritic polyamines, which are in the 6–10 nm range. An alternative explanation for this optimum weight range is that the optimal complex size is reached by polyamines with molecular weights in the 6000–25000-g mol^{-1} range.

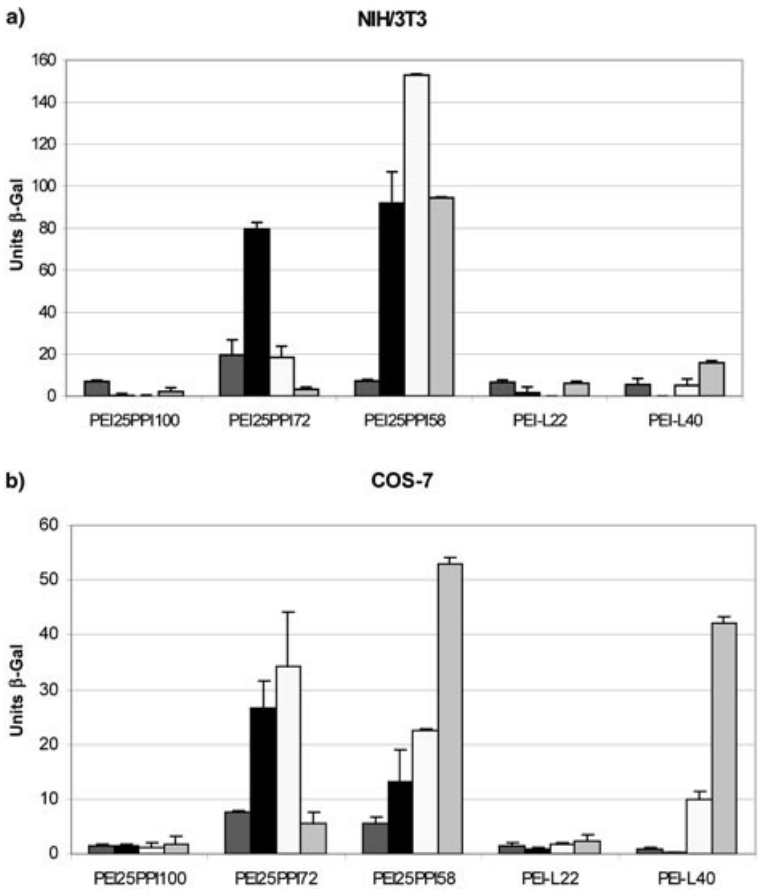


Figure 5. *Transfection efficiencies of $PEI_{25}PPI$ dendrimers with different degrees of branching (DB = 58, 72, 100%) and linear PEI (PEI-L, DB = 0%) molecules with two different molecular weights in a) NIH/3T3 and b) COS-7 cells. Several amounts of polyamine (1–4 μg (left to right) per well of a 96-well plate) were used for the transfection experiments. The results shown represent the average of the efficiencies determined for two replicates that have been performed in parallel. NIH/3T3 cells reached 147 units β-Gal after transfection with SuperFect®. The transfection efficiencies of the PEI/PPI analogues were all at least a factor of four lower than that obtained with SuperFect® in COS-7 cells depending on the analogue used.*

Influence of flexibility (degree of branching)

Functionalized PEIs (PEI/PPI) **3** with various degrees of branching were analyzed (Figure 5a, b). Functionalization was achieved by Michael addition of acrylonitrile to PEI and reduction of the nitrile groups in a second step to obtain the corresponding amine **3** (see above). The PEI used has a molecular weight of 25000 g mol^{-1} and the degrees of functionalization of the N–H bonds in the analogues (ratio acrylonitrile/N–H in the reaction mixture) were 35, 54, and 100%, which gave DBs of 58, 72, and 100%, respectively (see Figure 2b). For comparison, linear PEIs with molecular weights of 22000 g mol^{-1} and 40000 g mol^{-1} were analyzed. We were surprised to find that both the linear PEI analogues and the fully branched polymer ($PEI_{25}PPI_{100}$) have very low transfection efficiencies. The two polymers with a lower degree of branching ($PEI_{25}PPI_{58}$ and $PEI_{25}PPI_{72}$) performed much better, both in NIH/3T3 cells and in COS-7 cells (Figure 5a, b). Addition of greater amounts of the linear PEI with a molecular weight of 40000 g mol^{-1} resulted in a transfection efficiency comparable to those of $PEI_{25}PPI_{58}$ and $PEI_{25}PPI_{72}$ in COS-7 cells.

A significant increase in gene transfection efficiency can be seen in NIH/3T3 and CHO-K1 (data not shown) cells under optimal transfection conditions when the less-branched $PEI_{25}PPI_{58}$ dendrimer is used rather than $PEI_{25}PPI_{72}$. The difference was less pronounced in COS-7 cells. To evaluate this interesting observation in more detail, three different PEIs ($M_w = 5000$, 21000, and 25000 g mol^{-1}) were functionalized with acrylonitrile to obtain PEI_5PPI_{63}, $PEI_{21}PPI_{60}$, and $PEI_{25}PPI_{58}$, each with a DB of around 60% (Figure 6a, b).

The DNA transfection efficiency in NIH/3T3 increases when PEI cores with molecular weights of 25000 g mol^{-1} are used instead of lower-molecular-weight cores if other transfection parameters are optimal. This effect was also observed in COS-7 cells although to a smaller extent. In this cell line, use of $PEI_{25}PPI_{58}$ resulted in the best transfection efficiencies. No obvious cytotoxicity was observed microscopically for any of these polymers, in contrast to pure PEI with a similarly high molecular weight (see the first set of experiments, Figure 4a, b). The results show that a degree of branching of about 60% provides the dendritic architecture with the optimal flexibility for efficient interaction with the DNA backbone and still allows the polymer to swell after protonation in the endosome (proton sponge effect), as has been discussed elsewhere for linear polyethylenimines.[4]

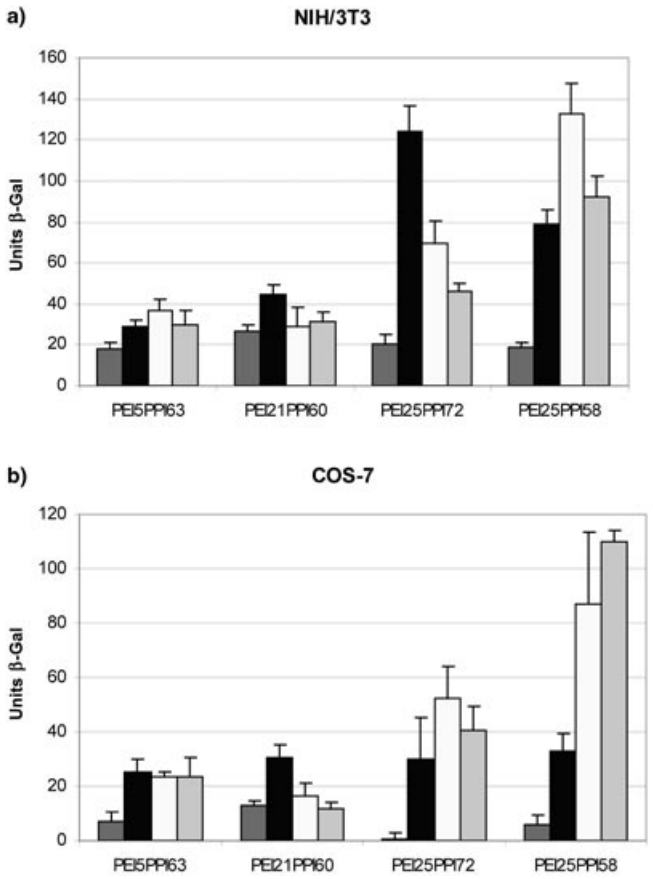


Figure 6. *Transfection efficiencies of PEI/PPI analogues with different degrees of branching and PEI cores with different molecular weights in a) NIH-3T3 b) COS-7 cells. Various amounts of polyamine (1–4 μg (left to right) per well in a 96-well plate) were used for the transfection experiment. The results shown represent the average of the efficiencies determined for two replicates that have been performed in parallel. After transfection of NIH/3T3 cells with SuperFect®, 125 units β-Gal were detected. SuperFect® transfections in COS-7 cells had efficiencies at least a factor of two higher than those achieved with the corresponding PEI/PPI analogues.*

Molecular weight dependence of the transfection efficiency of PAMAM analogues

In a third set of transfection experiments, PAMAM analogues (functionalized PEIs) **5** with two different core sizes were analyzed (PEI_5PAMAM and PEI_{21}PAMAM, Figure 7). It was not possible to control the DBs of these polymers through the degree of functionalization because the synthetic route used allows side reactions to occur even when the reagents are present in stoichiometric amounts. Two PEIs (M_w = 5000 and 21 000 g mol^{-1}) were used as starting materials and modified with a PAMAM analogue shell (see above) to obtain DBs in the 90–95% range. Depending on the cell line, these two investigated PEIs (M_w = 5000 and 21 000 g mol^{-1}) showed either comparable transfection efficiencies (NIH/3T3) or at least a factor of two lower transfection efficiencies than SuperFect®. No obvious cytotoxicity was observed microscopically for either of the PAMAM analogues.

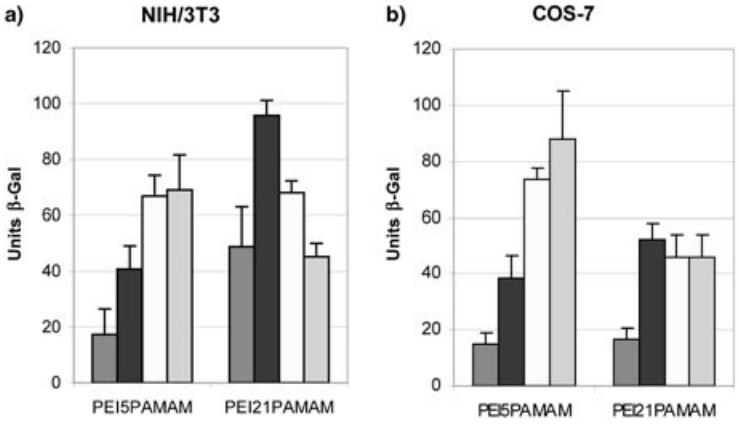


Figure 7. *Transfection efficiencies of PEI/PAMAM analogues with two different PEI-core molecular weights in a) NIH-3T3 and b) COS-7 cells. Various amounts of polyamine (1–4 μg (left to right) per well of a 96-well plate) were used for the transfection experiment. The results shown represent the average of the efficiencies determined for two replicates that have been performed in parallel. Transfection of NIH/3T3 cells with SuperFect® resulted in the presence of 109 units β-Gal in the cells. Transfection with PEI/PAMAM analogues in COS-7 cells was at least a factor of two less efficient than with SuperFect®, depending on the analogue used.*

These results suggest that a core molecular weight between 5000 and 21 000 g mol^{-1} should be used to obtain PAMAM analogue polymers that are suitable for DNA transfection in more sensitive cell lines (e.g. COS-7).

Conclusion

We have developed a simple synthetic route to dendritic polyamines with defined structures. We studied the influence of the size and degree of branching of the dendritic polymers on in vitro DNA transfection in several adherent cell lines. Both parameters influence the transfection efficiency and the cell toxicity. Depending on the cell line investigated, the optimum molecular weight for the PEI core is in the range 6000–25 000 g mol^{-1}, although cytotoxicity generally rises with increasing core size. Polymers with a lower degree of branching and more flexibility were also synthesized. Treatment of a 25 000 g mol^{-1} PEI with a reaction mixture containing acrylonitrile/NH in a ratio of 30–50%, followed by reduction of the nitrile groups, gave PEI/PPI polymers with DBs in the range of 50–70% and good gene transfection efficiencies. A higher DB results in a significantly lower gene transfection efficiency as a result of the lower flexibility of the structure, which might lead to a decreased swelling capability in the endosome after protonation (reduced proton sponge effect). As a side effect of functionalization, the cytotoxicity was lowered in the cell lines tested. Fully branched PEI/PPI polymers and linear PEIs (22 000 g mol^{-1} and 40 000 g mol^{-1}) have low transfection efficiencies. Smaller PEI/PPI polymers with molecular weights of 5000 and 21 000 g mol^{-1} and DBs of 63% or 60%, respectively, are not as efficient as larger PEI/PPI polymers (25 000 g mol^{-1}) with a DB of 58%. These results, together with the data gathered for NIH/3T3 and COS-7 cells, indicate that maximum transfection efficiency is obtained at a degree of branching of around 60% and a PEI-core molecular weight of

25 000 g mol^{-1}. PAMAM functionalization of PEI (5000 and 21 000 g mol^{-1}) also leads to polymers with little or no cytotoxity in the cell lines investigated.

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Keywords: dendrimers · DNA transfection · gene delivery · hyperbranched polyamines · polymers

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