Simple Modifications of Branched PEI Lead to Highly Efficient siRNA Carriers with Low Toxicity

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Polymer carriers like PEI which proved their efficiency in DNA delivery were found to be far less effective for the applications with siRNA. In the current study, we generated a number of nontoxic derivates of branched PEI through modification of amines by ethyl acrylate, acetylation of primary amines, or introduction of negatively charged propionic acid or succinic acid groups to the polymer structure. The resulting products showed high efficiency in siRNA-mediated knockdown of target gene. In particular, succinylation of branched PEI resulted in up to 10-fold lower polymer toxicity in comparison to unmodified PEI. Formulations of siRNA with succinylated PEI were able to induce remarkable knockdown (80% relative to untreated cells) of target luciferase gene at the lowest tested siRNA concentration of 50 nM in Neuro2ALuc cells. The polyplex stability assay revealed that the efficiency of formulations which are stable in physiological saline is independent of the affinity of siRNA to the polymer chain. The improved properties of modified PEI as siRNA carrier are largely a consequence of the lower polymer toxicity. In order to achieve significant knockdown of target gene, the PEI-based polymer has to be applied at higher concentrations, required most probably for sufficient accumulation and proton sponge effects in endosomes. Unmodified PEI is highly toxic at such polymer concentrations. In contrast, the far less toxic modified analogues can be applied in concentrations required for the knockdown of target genes without side effects.

INTRODUCTION

Since double-stranded RNA molecules were identified as the mediator of gene silencing in *C. elegans* (*1*), RNA interference (RNAi) has become a powerful strategy for intervention also in mammalian cell processes and treatment of various diseases, due to its ability to silence gene expression in a sequence-specific manner (*2–4*).

Polycations such as polyethylenimine (PEI)[1] have been introduced as nonviral gene carriers with a capability of forming stable complexes by electrostatic interactions with nucleic acids. The polyplex formation leads to improved protection of the genetic material from enzyme-mediated digestion and enhanced intracellular delivery. PEI, although it is known to be a powerful agent in plasmid-based gene delivery (*5, 6*), has been shown to be less effective in siRNA-mediated gene silencing (*7, 8*). Moreover, it is known to be toxic when applied at higher doses. The toxicity is mainly associated with a strong positive charge of this polycation, which leads to strong interaction of PEI with cell surfaces and their damage. Therefore, modifications of polymeric backbone that reduce the positive charge of PEI might be useful in order to reduce the toxicity of the polymer.

Several approaches have already been investigated in this direction. Different polymeric forms of PEI are available, the linear form (obtained via polyisoxazoline precursors) or the branched form (obtained from acid-catalyzed polymerization of aziridine), both in various degrees of polymerization that influence their biological activities (*9–12*). Backbone modification of linear PEI 22 kDa, which on its own is one of the most potent transfection agents (*6*), have reduced the high gene transfer activity (*13*). In fact, it was demonstrated that the high activity depends on complete removal of propionamide residues from the polyisoxazoline precursor polymer (*14*). Linear PEI22 on its own has limited efficiency in siRNA delivery (*7*); the activity however can be enhanced with concatemerizing "sticky" siRNA (ssiRNA) applied for polyplex formation (*15*).

Experiences with branched PEI 25 kDa gave different trends. Interestingly, partial acetylation of PEI amino groups was shown to enhance gene transfer activity (*16*). N-Acylation of PEI lowered its toxicity (*17*). Also, conjugation of acetate, butanoate, and hexanoate to branched PEI at low degree of substitution (below 25%) resulted in moderate improvement of transfection activity as demonstrated by Putnam and colleagues (*18*). The same research group compared different (linear and branched) PEIs for siRNA transfer activity; only branched PEI within a narrow window of conditions was able to mediate in vitro gene silencing in a (HR5-CL11) cell line (*8*). Reducing the surface charge of polyplexes by PEGylation of PEI positively affected the siRNA delivery capacity in the absence (*19*) or in the presence of a cell targeting molecule (*20*).

In this work, using relatively simple modification strategies, we were able to produce a number of branched PEI derivates, which were aimed at screening the positive charge of the polymer. In particular, negative charges were introduced into the polymer backbone by modification with succinic anhydride or Michael addition of ethyl acrylate followed by ester hydrolysis. Alternatively, amino groups were modified by acetylation or addition by ethyl acrylate. As was expected, all the products showed reduced toxicity in comparison to unmodified PEI. Moreover, we found that several modifications were able to greatly improve the siRNA/PEI formulation characteristics and

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[1] Abbreviations: PEI, polyethylenimine; EA, ethyl acrylate; EtdBr, ethidium bromide; RLU, relative light units.

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silencing activities, thereby presenting easy access to promising siRNA transfection reagents.

EXPERIMENTAL SECTION

Materials. Ethyl acrylate (EA), acetic anhydride, and succinic anhydride were obtained from Sigma (Munich, Germany). The plasmid pEGFPLuc (Clontech Laboratories, Heidelberg, Germany) containing a CMV promoter-driven fusion of the genes encoding for enhanced green fluorescent protein and luciferase was used for generation of stably transfected cells. Branched PEI with an average molecular weight of 25 kDa (PEI) was obtained from Sigma-Aldrich (Munich, Germany). PEI was used as a 1−10 mg/mL stock solution neutralized with HCl. All other chemicals were purchased by Sigma-Aldrich (Munich, Germany). Cell culture media, antibiotics, and fetal calf serum (FCS) were purchased from Invitrogen (Karlsruhe, Germany). Ready to use siRNA duplexes were purchased from MWG-Biotech (Ebersberg, Germany), namely, luciferase-siRNA; GL3 luciferase duplex; 5′-CUUACGCUGAGUACUUCGAdTdT-3′; control-siRNA; nonspecific control duplex with similar GC content as antiluciferase-siRNA; 5′-AUUGUAUGCGAUCG-CAGACdTdT-3′.

Modification of PEI by Ethyl Acrylate (EA). About 1 g of PEI was dissolved in chloroform. The solution was heated to 40 °C. The desired amount of acrylate was added to the solution with stirring. The reaction proceeded for at 40 °C. After 4 h, the chloroform was removed by rotary evaporator. The waxy product was dissolved in 15 mL water and neutralized by HCl to the pH 4−5 and freeze-dried. The modification degree was analyzed by ^{1}H NMR in D_2O on a Jeol JNMR-GX400 (400 MHz) or Jeol JNMR-GX500 (500 MHz) spectrometer from the ratio between the peaks of PEI (δ 2.6−3.6 ppm) and methyl acrylate (δ = 1.3 ppm). The modification degree was expressed as a number of modifications per monomer unit of PEI × 100%. According to FTIR spectra recorded on a Jasco FT/IR-410 spectrometer (Jasco Labor- and Datentechnik GmbH, Germany), a pronounced ester peak (1730 cm^{-1}) was detected. No significant amide band (1652 cm^{-1}) was found.

Synthesis of Acetylated and Succinylated PEI. PEI (0.5 g) was dissolved in 8.5 mL of water and 1.5 mL of NaCl solution (3 M) and then adjusted to pH 5 using 1 M HCl. The desired amounts of acetic or succinic anhydride were dissolved in DMSO and added dropwise to PEI solution. The reaction was carried out at room temperature for 3 h. The crude products dialyzed (18 000 cutoff, Spectra/Por membrane) first against 0.25 M NaCl solution to remove unreacted acetate or succinate, and then twice against water at 4 °C to remove salt. After the dialysis, the aqueous solution was lyophilized. The modification degree was analyzed by ^{1}H NMR in D_2O from the ratio between the peaks of PEI (δ 2.6−3.6 ppm) and methylene of succinate (δ = 2.5 ppm) or methyl of acetate (δ = 2.1 ppm). The modification degree was expressed as a percentage of modified amines of PEI under the assumption that each amine is able to react.

Synthesis of Propionic Acid Modified PEI by Hydrolysis of EA Modified PEI. About 0.3 g of PEI-EA polymer was dissolved in 1 M HCl. The solution was incubated at 40 °C for 3 days. The final product was dialyzed against water (18 000 cutoff, Spectra/Por membrane) and freeze-dried. The extent of reaction was controlled by ^{1}H NMR in D_2O. No ethyl peak was found for all samples, indicating complete hydrolysis of the ester bond. Neither ester (1730 cm^{-1}) nor amide (1652 cm^{-1}) peak was detected by FTIR.

Polyplex Formation. In all studies, the composition of polyplexes was characterized by the w/w ratio of the polymer to nucleic acid in the mixture. The weight of unmodified PEI represents a weight of the polymer in base form. The weight of modified PEI represents the weight of lyophilized product, which was previously neutralized to pH of about 4−5 and, therefore, includes the mass of counterions (chloride). Formulations for pDNA and siRNA delivery were prepared in HBG (20 μL) as follows: First, different concentrations of the nucleic acid and PEI derivatives were diluted at various polymer/nucleic acid ratios (w/w: weight/weight) in separate tubes in HBG (HEPES-buffered glucose solution; 20 mM HEPES, 5% glucose). Then, the HBG solution of PEI was added to the HBG solution of the nucleic acid, mixed, and incubated for 30 to 40 min at room temperature to form stable polymer/nucleic acid complexes.

EtBr Exclusion Assay. The ability of the products to condense DNA or siRNA was studied using an ethidium bromide (EtBr) assay. Polymer solution was added stepwise to the solution of nucleic acid (10 μg/mL) in HBG containing EtBr (0.4 μg/mL). After each step, fluorescence intensity was monitored (λ_{ex} = 510 nm, λ_{em} = 590 nm). The fluorescence intensity of the EtBr solution in the presence of free nucleic acid corresponded to 0% condensation, whereas the fluorescence intensity without nucleic acid corresponded to 100% condensation.

Polyplex Stability Assay. Polyplexes were mixed with DNA or siRNA in HBG at a final concentration of 20 μg/mL of nucleic acid and a 1:1 w/w ratio. The particle sizes were monitored by dynamic laser light scattering using a Malvern Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK). After this, 3 M NaCl solution was added stepwise to the polyplex solution. After each step, the correlation functions and scattering intensities were monitored. The dissociation of polyplexes led to the drop of scattering intensity to the level of the salt buffer and changes in correlation function (Supporting Information Figure 1). The NaCl concentration at which the dissociation of the polyplexes occurred was related to the stability of the polyplexes.

Cell Culture. For pDNA and siRNA delivery experiments, mouse melanoma cells B16F10, mouse glioblastoma cells Neuro2A, Neuro2A/EGFPLuc cells, and HUH7/EGFPLuc cells stably transfected with the EGFPLuc gene (see below) were used. B16F10 cells and Neuro2A/EGFPLuc cells were grown in Dulbecco's modified Eagle's medium (DMEM) and HUH7/EGFPLuc were grown in DMEM/Ham's F-12 medium, whereby all media were supplemented with 10% FCS, 4 mM stable glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were seeded 24 h prior to pDNA or siRNA delivery using 5000 cells per well in 96-well plates for analysis of protein expression.

For the generation of stably transfected cells, the vector pEGFPLuc (Clontech) was linearized using the restriction enzyme Dra III, and cells were then transfected with this linearized vector using linear polyethylenimine 22 kDa (PEI 22lin; Euromedex, Souffelweyersheim, France).

Following gene transfer, Neuro2A or HUH7 cells were incubated in fresh medium for 72 h prior to selection of the stably transfected cells with 200 to 2000 μg/mL G418 (geneticine; Invitrogen, Karlsruhe, Germany). After several days, surviving cells were seeded at low density into 6-well plates in order to generate separate colonies. Single cell clones were then isolated and expanded. The generated clones were analyzed for the percentage of EGFP-positive (EGFPLuc stably transfected) cells by flow cytometry. Clones with the highest number of EGFP-positive cells were then further selectively grown up under the above-described selective conditions, and this procedure was repeated until all cells were positive for EGFP. These stably transfected cells expressing approximately 60 ng luciferase per million cells were then used for siRNA delivery experiments.

In Vitro Gene Transfer. In vitro transfection efficiency of the polymers was evaluated in B16F10 or Neuro2A cells using

pEGFPLuc (200 ng pDNA per well). In all experiments, pDNA delivery was performed in 96-well plates with 5000 cells (B16F10) or 10 000 cells (Neuro2A) per well in triplicates. Cells were seeded 24 h prior to transfection and then medium was replaced with 80 μL fresh growth medium containing 10% FCS. Transfection complexes for pDNA delivery (20 μL in HBG) at different w/w ratios were added to each well and incubated at 37 °C. After 4 h, the medium was replaced by fresh growth medium. The cells were treated with 50 μL cell lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 2 mM DTT, 10% glycerol, 1% Triton X-100) 24 h following pDNA transfection. Luciferase activity in cell lysate (20 μL) was measured using a luciferase assay kit (100 μL Luciferase Assay buffer, Promega, Mannheim, Germany) on a luminometer for 10 s (Lumat LB9507 instrument, Berthold, Bad Wildbad, Germany).

In Vitro Gene Silencing with siRNA. In vitro gene silencing experiments were performed in stably transfected Neuro2A/EGFPLuc and HUH7/EGFPLuc cells using LucsiRNA and MutsiRNA as a control. In all experiments, siRNA delivery was performed in 96-well plates with 5000 cells per well in triplicate. Cells were seeded 24 h prior to transfection, and then medium was replaced with 80 μL fresh growth medium containing 10% FCS. Transfection complexes for siRNA delivery (20 μL in HBG) at different w/w ratios were added to each well and incubated at 37 °C without medium change. Cells were washed with phosphate-buffered saline (PBS) and treated with 50 μL cell lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 2 mM DTT, 10% glycerol, 1% Triton X-100) 48 h following siRNA transfection. Luciferase activity in cell lysate (20 μL) was measured using a luciferase assay kit (100 μL luciferase assay buffer, Promega, Mannheim, Germany) on a luminometer for 10 s (Lumat LB9507 instrument, Berthold, Bad Wildbad, Germany). The relative light units (RLU) were related to untreated control cells.

Cytotoxicity Assay. Neuro2A cells were seeded into 96-well plates at a density of 1×10^4 cells/well. After 24 h, the culture medium was replaced with 100 μL/well of serial dilutions of polymer stock solutions in growth medium. Alternatively, transfection complexes for pDNA delivery (20 μL in HBG) at different C/P ratios were added. All studies were made in triplicate. After 4 h incubation, the medium was replaced by the fresh one.

After 24 h, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, Germany) was dissolved in phosphate buffered saline at 5 mg/mL, and 10 μL aliquots were added to each well reaching a final concentration of 0.5 mg MTT/mL. After an incubation time of 1 h, unreacted dye with medium was removed. The purple formazan product was dissolved in 100 μL/well dimethyl sulfoxide and quantified by a plate reader (Tecan, Groedig, Austria) at 590 nm with background correction at 630 nm. The relative cell viability (%) relative to control wells containing cell culture medium without polymer was calculated by [A] test/[A] control $\times$ 100.

RESULTS

Synthesis of the Polymers. The strategies for modification of branched PEI 25 kDa are presented in Figure 1. In EA and PROP series, the primary amines of PEI were transformed to the secondary amines by Michael addition. In AC and SUC series, the primary amines were acetylated to amides. Additionally, PROP and SUC series have carboxylic group in the structure.

Syntheses and characterization are described in Materials and Methods and Supporting Information. PEI was modified with ethyl acrylate via Michael addition performed at 40 °C and relatively short incubation time. Table 1 shows the degrees of modification (12−31%) depending on the ratios in the feed.

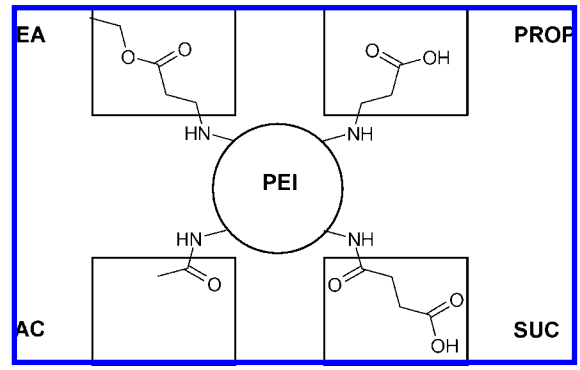

Figure 1. Strategies for modification of branched PEI.

Table 1. Composition of Modified PEIs[a]

polymer	reagent/amine*100%, feed	modification degree, %
PEI-EA 12	13%	11.5%
PEI-EA 18	26%	17.6%
PEI-EA 31	52%	31%
PEI-PROP 12	n.a.	11.5%
PEI-PROP 18	n.a.	17.6%
PEI-PROP 31	n.a.	31%
PEI-AC 12	10%	12.3%
PEI-AC 22	20%	21.8%
PEI-SUC 9	10%	8.9%
PEI-SUC 19	20%	19.4%

[a] The nomenclature of the polymers is expressed as follows: PEI-*X Y*, where *X* represents the reagent by which PEI was modified (EA, ethyl acrylate; SUC or AC, succinic or acetic anhydride, respectively) or functional group (PROP for 3-propionic acid) and *Y* represents the modification degree of the amines measured by NMR.

Longer incubation times are known to cause aminolysis of ester bond and lead to cross-linking of PEI (*21*). No amide peak was found on FTIR spectra of all polymer samples of PEI-EA series (data not shown). Therefore, no aminolysis was observed, excluding the cross-linking of PEI. No double bond peak was present in ^{1}H NMR for all samples, indicating the complete evaporation of unreacted acrylate during freeze-drying procedure and its absence in the final products.

The 3-propionic acid-modified B-PEI (PEI-PROP) was generated by acid hydrolysis of PEI-EA polymers. Complete hydrolysis was verified by disappearance of the ester protons in the NMR spectra and characteristic FTIR-band at 1730 cm^{-1}. The degrees of PEI modification with carboxylic groups were assumed to be the same as in nonhydrolyzed precursor polymers. An absence of the amide peak on FTIR spectra (1652 cm^{-1}) reveal no significant cross-linking during hydrolysis.

The modification of PEI with acetic anhydride and succinic anhydride was performed in the presence of salt in the reaction solution to avoid precipitation of the polymer. The purification of polymers via dialysis was performed against salt buffer to ensure complete exchange of acetic or succinic acid, respectively, against chloride. At low reagent/amine feed ratios (10−20%), nearly quantitative modification of PEI was achieved (Table 1).

The yields were typically 80−120%. The neutralization of the products and, consequently, the weight gain due to counterions (chloride), which was not taken into account by calculation of theoretical yields, was the reason for some of the yields being above 100%.

The pH titrations of the polymers demonstrated that the pH5 to pH7 buffer capacity did not significantly change after modification (data not shown).

Toxicity of the Polymers. The cell culture cytotoxicity profiles of modified PEIs (Figure 2) are shifted to higher polymer concentrations in comparison to PEI, demonstrating a

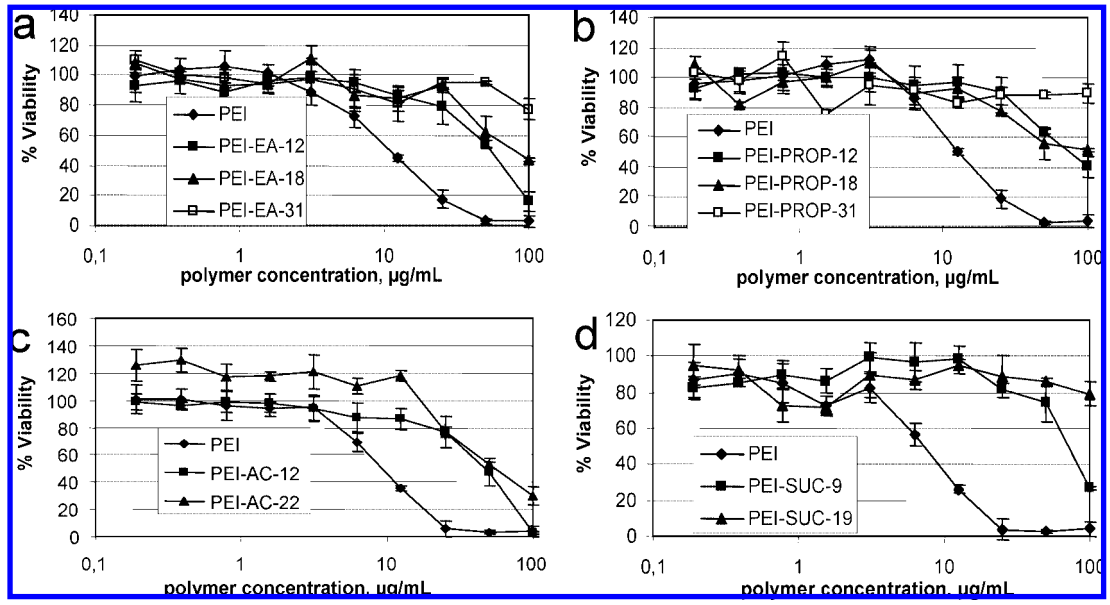

Figure 2. Cell viability of Neuro2A cells as a function of concentration of modified PEI polymers in the medium. (a) PEI-EA series, (b) PEI-PROP series, (c) PEI-AC series, (d) PEI-SUC series.

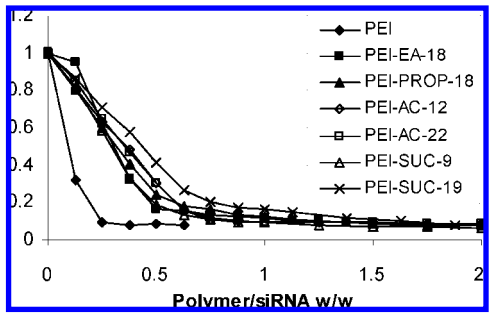

Figure 3. EtBr exclusion assay of the polymers with siRNA in HBG buffer.

decrease in toxicity. Relatively soft neutral modifications (EA and AC) have shown only moderate improvement of cell viability, whereas the incorporation of negative charges on PEI led to polymers with far less toxicity.

Chemical and Biophysical Properties of Polyplexes. In salt-free buffer, all polymers were effective in binding of siRNA (Figure 3) as well as DNA (data not shown). In comparison to PEI, the binding required higher polymer to siRNA and DNA (w/w) ratios. Such a difference is caused by the fact that for modified PEI w/w ratio was calculated from the base form. Modified PEIs were weighed in neutralized form; therefore, the counter ions (chloride) markedly contribute to mass per charge ratio. Nevertheless, at w/w ratio of 2 even the polymers with a high degree of modification show the reduction of EtBr fluorescence to less than 10%. No significant difference in binding between products of different modifications was observed in this assay. Agarose gel electrophoresis data confirmed these results (data not shown).

The analysis of the polyplex solutions mixed in HBG at w/w ratios of 2 and 4 by photon correlation spectroscopy did not show the formation of nanoparticles in the usual 30−300 nm range of standard DNA/PEI polyplexes. The scattering intensity from polyplex solution at a concentration of 5 μg/mL based on siRNA was close to that of HBG buffer, which indicates very small polyplex sizes and low aggregation number of the polymer chains. Such behavior of siRNA/PEI polyplexes has recently been shown by Meyer et al. (*22*) where particle sizes of around 25 nm were detected by fluorescent correlation spectroscopy.

The increase of the salt concentration in this solution to 150 mmol NaCl led to continuous aggregation of the initial polyplexes and formation of nanoparticles (around 500 nm after 30 min) in the case of modified PEI. Unmodified PEI without siRNA did not show any aggregation at physiological saline.

Plasmid-Based Gene Transfer. Most of the polymers were found to be effective gene transfer agents and showed transfection efficiencies comparable to branched PEI on B16F10 and Neuro2A cells (Supporting Information Figure 2). Only PEI-PROP-31 displayed relatively low efficiency. All others showed transfection efficiencies comparable to PEI. The polyplexes generated with the modified PEIs induce much less toxicity than PEI. As can be seen from Supporting Information Figure 2b,c, at w/w ratio of 4 the cell viabilities are above 80% for the modified PEI and only 20−40% for the unmodified PEI.

Gene Silencing with siRNA. Modified PEIs were found to be good DNA transfection agents. However, it is known from literature that efficient carriers of plasmid DNA are not necessarily effective for siRNA delivery (*23*). Linear polyethylenimine 22 kDa and branched polyethylenimine 25 kDa (PEI), the powerful DNA delivery agents, showed only limited efficiency in siRNA delivery (*7, 8*). Therefore, the modified PEIs were tested for their efficiency in siRNA delivery using a neuroblastoma cell line stably transfected with a luciferase marker gene. The results are presented in Figure 4. Luciferase activities were determined for cells treated either with LUC siRNA (directed against the target mRNA) or with a control siRNA (Mut siRNA), and compared with the luciferase activity of untreated control cells. Knockdown effects are real when expression decreases with Luc siRNA but not Mut siRNA. If luciferase expression decreases with both target and control siRNA, "knockdown" is mainly due to unspecific carrier toxicity. siRNA formulations with PEI were not able to facilitate knockdown of reporter gene without toxicity. High toxicity was observed for the formulations above w/w ratio of 1, indicated by decrease of luciferase level for formulations with mismatched siRNA.

Most modified PEIs (except PEI-PROP-31 and PEI-SUC-19) showed up to 80−90% of luciferase knockdown, when formulated with Luc-siRNA. The effect cannot be attributed to the toxicity of polyplexes, since the luciferase level by the formulations with mismatched siRNA was close to the control.

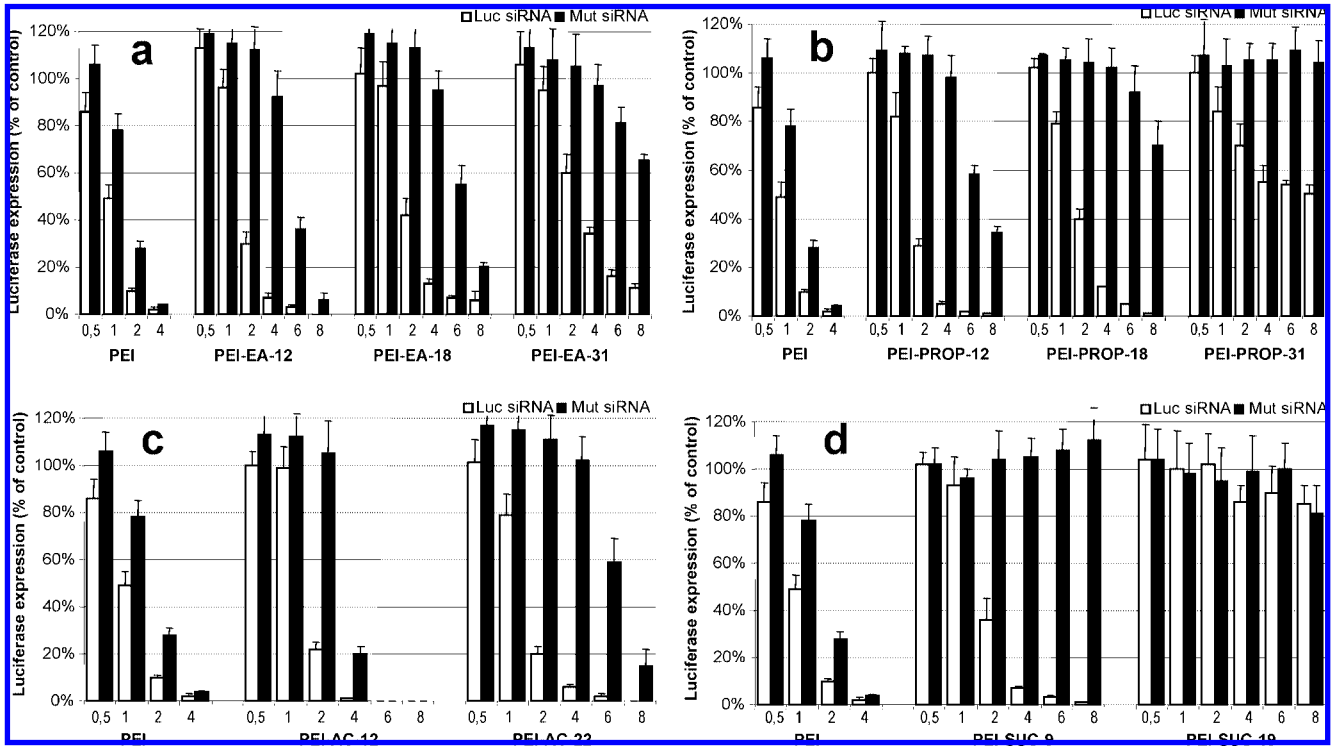

Figure 4. siRNA delivery to Neuro2A/EGFPLuc cells using modified PEI: (a) EA series, (b) PROP series, (c) AC series, (d) SUC series. The numbers on the *x*-axis represent the polymer/siRNA w/w ratios. The final siRNA concentration in culture medium was 380 nM. White bars, LucsiRNA; black bars, MutsiRNA. Luciferase expression levels are presented in % relative to untreated control cells. Luciferase expression was measured 48 h after transfection without change of the medium.

The best knockdown was achieved with PEI-SUC-9. Being nontoxic at all studied mixing ratios, it showed the gene silencing to almost less than 10% relative to the control. Further increase of the negative charge on PEI led to inactivation of this polymer; PEI-SUC-19 did not show any gene knockdown at all w/w ratios. Interestingly, PEI-EA-31 showed significant knockdown at w/w of 4 and 6, whereas the hydrolyzed analogue PEI-PROP-31 was inactive. Thus, low amounts of negatively charged groups on the polymer were able to improve the efficiency of PEI in siRNA delivery, whereas high amounts of negative charges resulted in nontoxic but also ineffective polymers.

Even with lower amounts of siRNA significant knockdown was achieved, if the polymer amount remained on the same level (Figure 5). At concentration of the polymers in transfection medium of 20 μg/mL and siRNA concentration of 50 nM, it was possible to achieve 50−80% knockdown of the luciferase gene. Decrease of siRNA concentration led to a certain increase of the toxicity of the formulations with most of the polymers, which was caused by an increasing amount of uncomplexed PEI in the transfection medium. Only PEI-SUC-9 was able to show up to 80% knockdown without any sign of toxicity at 50 nM siRNA concentration. Unmodified PEI at concentration of 2.5 μg/mL was taken as a control. As is seen, PEI was not efficient at any siRNA concentration. Higher concentrations showed unspecific knockdown due to the toxicity of the carrier.

PEI-SUC-9, as the most promising candidate, was additionally tested for siRNA delivery to another stably transfected cell line HUH7Luc (Supporting Information Figure 3). Also on this cell line, high efficiency and low toxicity of this carrier were demonstrated.

Bolcato-Bellemin et al. (*15*) postulated that the limited efficiency of PEI/siRNA formulations might be caused by low binding affinity of polycation to nucleic acid. Therefore, if the binding to PEI could be increased, the formulation with siRNA would gain efficiency. The modifications of PEI used in the present paper were not expected to increase the binding with siRNA. Nevertheless, several of the modified polymers showed strongly improved siRNA activity in comparison to that of PEI. To study this discrepancy and further define the influence of PEI modification on the binding between polymer and siRNA, the stability of the polyplexes against dissociation induced by salt was studied.

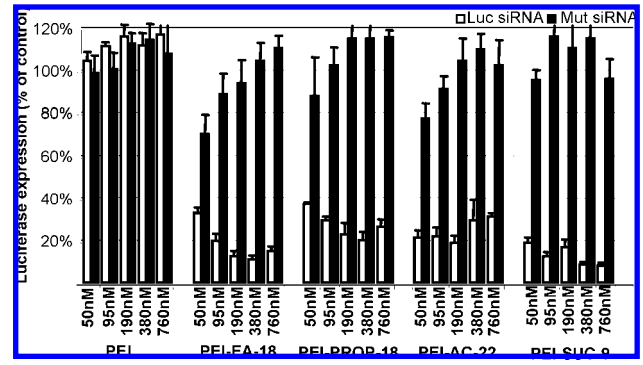

Figure 5. siRNA delivery to Neuro2A/EGFPLuc cells using modified PEI. The numbers on the *x*-axis represent the concentration of siRNA in culture medium. The concentration of PEI-EA-18, PEI-PROP-18, PEI-AC-22, and PEI-SUC-9 was kept constant at 20 μg/mL. The concentration of unmodified PEI was kept at 2.5 μg/mL. White bars, LucsiRNA; black bars, MutsiRNA. Luciferase expression levels are presented in % relative to untreated control cells. Luciferase expression was measured 48 h after transfection without change of the medium.

Electrostatic interactions between polycations and nucleic acids are largely responsible for the stabilization of polyplexes. Contrary to long nucleotide sequences (like plasmids), for relatively short oligomers the stabilization is expected to be dependent on the number of charges per nucleic acid molecule. Low molecular salt is able to screen the electrostatic interactions and, at certain concentration, cause the dissociation of poly-

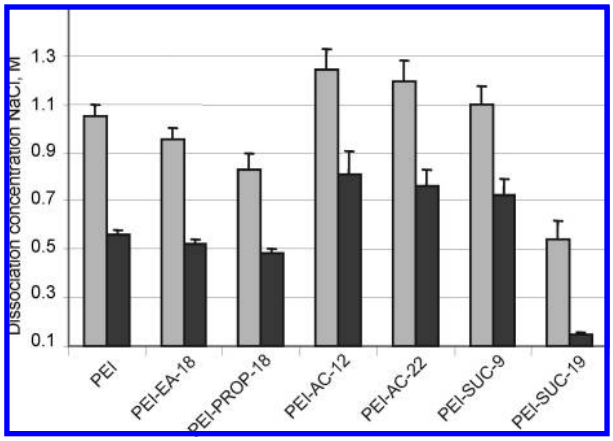

Figure 6. NaCl concentrations required for dissociation of polyplexes: gray bars, polyplexes with DNA; black bars, polyplexes with siRNA. For details, see Experimental Section and Supporting Information. Complexes were formed at concentration of 20 ug/mL nucleic acid and 1/1 w/w ratio.

plexes. The salt concentration required for the dissociation could be attributed to the stability of polyplexes. Photon correlation spectroscopy was used to detect the dissociation point. The polyplexes formed in salt-free buffer were titrated with NaCl. After each step, dynamic light scattering measurements were performed. Addition of NaCl to the polyplex solution led to aggregation of small polyplex particles, increase of scattering intensity, and shift of the correlation function to the slow-motion range (Supporting Information Figure 1). Further increase in NaCl concentration led to the dissociation point, where the scattering intensity drops to the level of NaCl buffer. The residual correlation is mainly caused by the statistically low number of foreign particles in the solution (i.e., dust) (Supporting Information Figure 1).

The NaCl concentrations required for the dissociation of the polyplexes with DNA and siRNA are presented in Figure 6. As expected, polyplexes with DNA dissociate at near two times higher salt concentration than polyplexes with siRNA. However, no correlation between the stability and knockdown efficiency within siRNA polyplexes was found, since all series of PEI modifications were found to show the efficient knockdown of the luciferase gene. According to the stability data, EA and PROP series showed lower stability than PEI, whereas in case of AC and SUC series, the stability was higher. The same dependence could be also attributed to the polyplexes with DNA. The reasons for the increased stability of the polyplexes in AC and SUC series are not clear. Additional stabilization of the polyplexes might be mediated through the hydrogen bonding between the amides in these structures and the nucleic acids (*24*).

Interestingly, the PEI-SUC-19 was able to transfect the cells, but was completely inefficient in inducing knockdown with siRNA (Figure 4d). Data from Figure 6 explain this behavior. While being relatively stable with DNA, siRNA polyplexes of this polymer are unstable at physiological salt concentration and not able to induce knockdown.

DISCUSSION

In this study, a number of PEI-based polymers were synthesized, which showed significantly lower toxicity in comparison to branched PEI. Since toxicity of PEI is mainly associated with the high positive charge of the polymer, the screening of PEI amines was performed in different ways. The Michael addition of ethyl acrylate (EA series) represents a relatively soft modification, which results in transformation of primary amines of PEI to secondary. Also, a bulky EA group is introduced to the structure, which could represent a steric barrier for interaction of PEI with negatively charged surfaces. The AC modification reduces the positive charges of PEI due to acetylation of primary amines. In the PROP series, the transformation of primary to secondary amines was combined with the introduction of negatively charged proionic acid residues. Finally, SUC series represent the strongest reduction of the positive charge of PEI by combination of acylation of primary amines and introduction of negative charges into the polymer.

As expected, the toxicity of the polymers was greatly reduced by the modifications. Even the softer PEI modifications (EA, AC) reduced the toxicity, although to a lower extent in comparison to the PROP and SUC series. These data fit very well with literature, where several modifications of branched PEI led to decreased charge and lower toxicity of the polymer (*17, 25*). The reduction of positive charge, however, might also reduce activity of polycation in nucleic acid transfer as previously reported (*13, 14, 19*). Within our series of modifications, only two products with the highest degrees of modification showed inactivation: PEI-PROP-31 for both DNA and siRNA transfer, and PEI-SUC-19 for siRNA transfer. Reduced polyplex stability due to the increased density of negative charges on the cationic polymer chain is presumably responsible for this. For example, polyplexes of siRNA and PEI-SUC-19 dissociated in physiological saline. All other modified PEI products were found to show transfection efficiency with DNA comparable to that of unmodified PEI. Importantly, in contrast to unmodified PEI, they were able to induce efficient siRNA-mediated knockdown. PEI-SUC-9 was found to be the most effective delivery agent for siRNA. Low toxicity and high efficiency in knockdown, also at low concentrations of siRNA, make this PEI modification very promising for in vitro delivery of siRNA.

Possible reasons for the low efficiency of unmodified PEIs in siRNA delivery despite their high efficiency in DNA transfer might be associated with the stability of the polyplexes. Requirements for delivery of DNA and siRNA are different. DNA transfer requires nuclear uptake with subsequent unpacking and release of the plasmid. Relatively strong binding of the carrier to the pDNA might prevent the unpacking and degradation of pDNA in the cytoplasm. In case of siRNA delivery, an effective dissociation from the carrier is required within the cytoplasm. Therefore, the modifications should aim at a lower affinity of the polymer to siRNA in comparison to DNA polypexes. Another hypothesis postulates that lower affinity of siRNA to the carrier as compared to plasmid DNA may be the reason for insufficient knockdown due to polyplex instability. Therefore, in order to achieve effective knockdown formulations, the stability of the polyplexes with siRNA should be increased.

Neither of the two hypotheses seems to be true. According to the data of the present investigation, the stability of the polyplexes with siRNA is lower in comparison to the polyplexes with DNA. Although all PEI modifications in this study were expected to reduce the electrostatic affinity to nucleic acids, certain series (AC and SUC) showed even higher stability of polyplexes in comparison to unmodified PEI. Taking into account that all modification types were able to induce significant knockdown, AC and SUC series do not fit the first hypothesis as well as EA and PROP series to the second. The stability of siRNA formulations could possibly play a role during in vivo applications; however, for in vitro efficacy the extent of polyplex stability has minor importance, unless the polyplex is sufficiently stable to survive in the culture medium.

The most probable reason for the improved properties of modified PEIs in siRNA delivery in comparison to unmodified PEI is the lower toxicity of the carrier, which enables higher

amounts of PEI for transfection. This hypothesis is also supported by DNA transfection data with unmodified PEI; the increase of w/w ratio from 0.8 to 4 led to increased transfection efficiency (and toxicity). Higher amounts of PEI in the formulation and subsequently in endosomal compartments may increase escape to the cytosol due to a stronger proton sponge effect (*26, 27*). Therefore, the transfection properties of PEI can be boosted by increasing the amount of polymer.

The concentration of PEI plays a major role for efficient siRNA delivery. At small concentrations, unmodified PEI is not able to facilitate significant knockdown (Figure 5). Higher concentrations lead to high toxicity of the carrier. In the case of modified PEIs, in particular, PEI-SUC-9, higher concentrations of PEI could be applied without toxicity. At these concentrations, even small amounts of siRNA could be applied to decrease the activity of the target gene. Therefore, the reduction of the toxicity has a really decisive role for the efficiency of siRNA formulations with modified PEI.

CONCLUSIONS

The amino group modifications of branched PEI with acetamide, β-propionic acid and ethyl ester, or succinic acid as presented in this study strongly increase the efficiency in siRNA delivery. For example, PEI-SUC can mediate knockdown at siRNA concentrations as low as 50 nM. The stability of polyplexes did not correlate with the efficiency. The most probable reason for the improved characteristics is the strongly reduced cytotoxicity of the modified PEI, which allows the administration of polyplexes formulated with a larger amount of polymeric carrier.

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Supporting Information Available: Additional information on dissociation of PEI polyplexes in NaCl, pDNA transfection efficiencies in B16F10 or Neuro2A cells, and siRNA delivery to HUH7/EGFPLuc cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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