# Proton Sponge Trick for pH-Sensitive Disassembly of Polyethylenimine-Based siRNA Delivery Systems

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Small interfering RNAs offer novel opportunities to inhibit gene expression in a highly selective and efficient manner but depend on cytosolic translocation with synthetic delivery systems. The polyethylenimine (PEI) is widely used for plasmid DNA transfection. However, the water-soluble PEI does not form siRNA polyplexes stable enough in extracellular media for effective delivery. We previously showed that rendering PEI insoluble in physiological media, without modifying drastically its overall cationic charge density, by simple conjugation with natural hydrophobic α-amino acids, can lead to effective siRNA delivery in mammalian cells. In here, we comprehensively investigated the mechanism behind the excellent efficacy of the leading PEIY vector. Our data revealed that the underlining proton sponge property is key to the effectiveness of the tyrosine−polyethylenimine conjugate as it may allow both endosomal rupture and siRNA liberation via an optimal pH-sensitive dissolution of the PEIY self-aggregates. Altogether, these results should facilitate the development of novel and more sophisticated siRNA delivery systems.

## INTRODUCTION

RNA interference (RNAi) is a sequence-specific and post-transcriptional gene silencing process present in animals and in plants and is mediated by 21−22nt long RNA duplexes, called small interfering RNA (siRNA) (*1*). This gene silencing mechanism appears to be particularly effective and holds great potential for decoding gene function and in gene-specific therapeutics (*2*). However, oligonucleotides do not diffuse freely across plasma membrane and imperiously depend on formulation within synthetic delivery systems (*3, 4*).

Nonviral delivery vehicles were initially developed for plasmid delivery (*5, 6*). They are generally cationic lipids or cationic polymers that interact electrostatically with the nucleic acid phosphate backbone to form stable complexes (*7, 8*). These cationic complexes in turn bind to anionic proteoglycans present on cell surfaces, enter cells within membrane-coated vesicles, and experience acidification on their road to degradative compartments. Escape from this pathway is required and relies on the incorporation of fusogenic lipids or endosomolytic functions within the complexes. Among the cationic polymers, PEI[1] is certainly the most used plasmid DNA transfection agent because its high buffering capacity in the pH range between 5.0 and 7.5 facilitates rupture of endosomal membranes via a "proton sponge" mechanism (*9, 10*). However, PEI was shown to be a poor siRNA delivery agent (*11*), especially in comparison to lipids (*12, 13*).

Numerous approaches and hypotheses have been investigated to create efficient polymer-based delivery vehicles dedicated to siRNA (*14*−*21*). While siRNA duplexes and genes share a similar anionic charge density, the reduced number of anionic charges of a siRNA duplex (40) in comparison to a plasmid DNA (average anionic charge of 7000) reduces the electrostatic cohesion of the soluble PEI with siRNA. Polyanionic proteoglycans present outside the cells and on the cell surfaces may then effectively displace PEI from the complexes, resulting in release of siRNA in the extracellular medium (*22*). No delivery and siRNA-mediated gene silencing can consequently occur.

Increased stability of oligonucleotide polyplexes may be performed by three means. siRNA duplexes could be artificially transformed into long structures, like plasmid DNA, by equipment with self-complementary and overhanging nucleotides (*22*). Oligonucleotides could be conjugated with cholesterol for enhanced anchorage to cationic micelles (*23*) or to cationic peptide so as to obtain an overall self-aggregating and cationic species (*24*). Finally, transformation of the water-soluble PEI into an insoluble molecule, similar to cationic lipids, could also favor overall siRNA delivery. We have previously reported that the latter approach was successful (*25*). Simple modification of the commercial branched 25 kDa PEI with various α-amino acids, at a modification degree of 30% per ethylenimine, maintains the proton sponge and siRNA binding properties of the vector, but confers self-aggregation and siRNA delivery aptitude to the polymers (Figure 1). The tyrosine-modified PEI (PEIY) appeared particularly effective relative to the other self-aggregating polymers as it led to more than 90% luciferase expression silencing versus only about 50% with the other ones. The effectiveness of the PEIY could not be explained by a molecular specificity of the tyrosine. Indeed, decreasing the content of the PEIY from 30% to 20% (PEIY20) diminishes the delivery potential, while an opposite trend is observed with

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[1] Abbreviations: PEI, polyethylenimine; BSA, bovine serum albumin; RLU, relative light units; FBS, fetal bovine serum; PBS, phosphate buffer saline; α-aa, α-amino acid; Boc, *tert*-butyloxycarbonyl; DLS, dynamic light scattering; NR, Nile Red; CTAB, cetyl trimethylammonium bromide; siRNA, small interfering RNA; Y, tyrosine; W, tryptophan; R, arginine; F, phenylalanine; L, leucine; RPMI medium, Roswell Park Memorial Institute 1640 medium.

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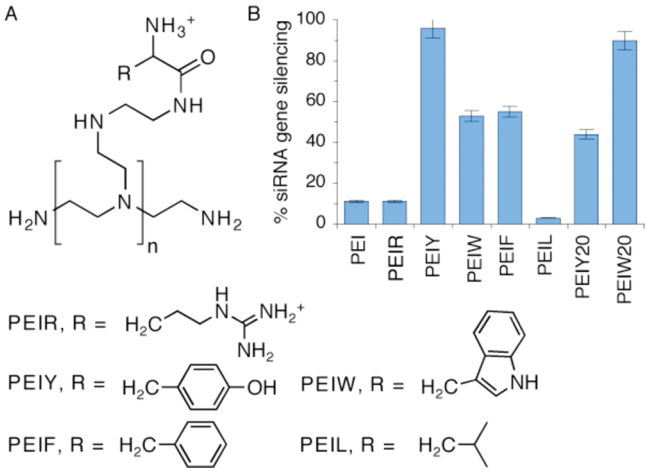


**Figure 1.** A. Chemical structure of the α-amino acid−polyethylenimine conjugates. B. siRNA delivery abilities of the various polymers in a model cell line, as judged by inhibition of a reporter gene expression. siRNA (10 nM) was delivered to A549Luc cells with 20 $\mu$M of the indicated polymer.

the tryptophan-modified PEI (PEIW) for which 20% amino acid content generated a polymer with almost the same efficiency as the PEIY.

In the present work, we comprehensively investigated the mechanism behind the excellent efficacy of the leading PEIY vector. We measured the hydrophobicity of several representative polymers using a fluorescent probe and evaluated their hemolytic, cytotoxic, self-aggregating, and siRNA delivery abilities. Results revealed that the acidic environment encountered in PEI-loaded endosomes can direct dissolution of tyrosine−polyethylenimine aggregates and mediate disassembly of siRNA polyplexes through protonation of the polymer.

## EXPERIMENTAL PROCEDURES

**Materials.** The amino acid-modified PEIs were prepared as previously described (*25*). Succinimidyl ester of N-Boc protected α-amino acids were purchased from Novabiochem, Merck KgaA (Darmstadt, Germany). Branched PEI (40,872−7, batch 09529KD-466; 25 kDa) was from Aldrich (St. Quentin, France). Water was deionized on a Millipore Milli-Q apparatus. All other chemicals were at least of analytical grade and were used as supplied. NMR spectra were performed on Bruker DPX 300 or 200 MHz spectrometers. HiPerfect (Qiagen) was used according to manufacturer's protocol. Before use, dialysis membranes were soaked in Milli-Q water (200 mL, 3 times, 8 h each) to remove preservatives.

**Synthesis of Fluorescently Labeled PEIY (Green-PEI).** 6-Carboxyfluorescein *N*-hydroxysuccinimide ester (2.4 $\mu$mol), freshly dissolved in methanol (1 mL), was added at once to a solution of PEIY (0.2 M in ethylenimine, 1.0 mL), which was first buffered to pH 7.0 by addition of 0.6 equiv NaOH. The solution was stirred in the dark overnight at room temperature and the solution subjected to dialysis using a SpectraPor 12−14 kDa membrane against methanol/water 1/1 mixture (0.1 L, 2 changes over a 48 h period), aqueous 100 mM HCl (0.1 L, 24 h), followed by water (0.2 L, 2 changes over a 48 h period).

**Size Measurement.** The apparent sizes were determined via dynamic light scattering measurements using a NanoZS apparatus (Malvern instruments, Paris, France) with the following specifications: sampling time = 90 s; refractive index of medium = 1.3402; refractive index of particles = 1.47; medium viscosity = 1.145 cP; and temperature = 25 °C. Data were analyzed using the multimodal number distribution software included with the instrument.

**Determination of the Polymer Hydrophobicity.** A stock solution of Nile Red was prepared in DMSO at a concentration of 0.4 mM and stored at −20 °C. Stock solutions of polymer were in water at a 0.2 M concentration in ethylenimine. Each polymer (2 $\mu$L) was first added to 1 mL RPMI solution to final concentration of 400 $\mu$M. Increment (0.2 $\mu$L) of the Nile Red solution was then added between measurements. The excitation wavelength was set at 540 nm (bandpass: 5 nm) and emission was recorded from 580 to 700 nm (bandpass: 5 nm) using a fluorescent spectrophotometer. Representative emission spectra were extracted from experiments containing 0.56 $\mu$M Nile Red.

**Determination of Polymer Aggregation pH.** Self-aggregation was determined by turbidity. Each polymer (HCl salt) was first solubilized in water at a 20 mM concentration (0.5 mL volume). The solution was then titrated by addition of NaOH solution (0.25 M, 1 $\mu$L increment) until occurrence of aggregate as seen by turbidity. pH of the solution was then measured with a calibrated pH meter. The turbid solution was then titrated with HCl (0.1 M, 1 $\mu$L increment) up to full dissolution and the pH measured again. For all polymers, the transition was relatively narrow and within 0.2 pH unit.

**Hemolysis Experiments.** For recovery from washing steps, sheep red blood cells (RBC) (Eurobio, Courtaboeuf, France) were centrifuged at 400 RCF for 10 min. Before experiments, cells were washed three times with NaCl aqueous solution (150 mM). RBC were then resuspended in phosphate buffer (100 mM, pH 7.4), prepared to be isoosmotic to the inside of a RBC and caused negligible hemolysis, and plated in 96 well plates to obtain $15 \times 10^6$ cells in 50 $\mu$L. 50 $\mu$L aliquots of polymer solutions at different concentrations, also prepared in the same phosphate buffer, were added to the erythrocytes and incubated for 2 h at 37 °C and 5% $CO_2$. The release of hemoglobin was determined after centrifugation at 700 RCF for 10 min by spectrophotometric analysis of the supernatant at 550 nm (Bio-Rad model 550, Marnes-la-Coquette, France). Complete hemolysis was achieved using deionized water yielding the 100% control value. The negative control was obtained by suspension of RBC in phosphate buffer alone. The experiments were performed in triplicate.

**siRNA Polyplexes Preparation.** PAGE-purified siRNA were purchased from Eurogentec (Seraing, Belgique) and stored at −20 °C as a 20 $\mu$M solution in RNase-free water as indicated by the manufacturer. The luciferase gene originating from pGL3 plasmid was silenced with a RNA duplex of the sense sequence: 5′-CUUACGCUGAGUACUUCGA. Untargeted pGL2luc RNA duplex was of sequence 5′-C**G**UACGC**G**GAA**A**UACUUCGA (bold marks showed mismatches by comparison to pGL3 sequence). Human papillomavirus 18 E6 oncoprotein from infected HeLa cells was silenced with a RNA duplex of sense sequence 5′-CUAACUAACACUGGGUUAU.

To prepare polyplexes, appropriate quantities of siRNA and polymer were separately diluted in RPMI medium (Eurobio, Courtaboeuf, France). After 10 min equilibration, the polymer solution was added to the siRNA solution. Then, the mixture was vortexed for 10 s and incubated at room temperature for 30 min.

**Determination of Polyplex Stability.** 50 pmol of siRNA and 100 nmol of polymer buffered to pH 6.0 were prepared either in RPMI (15 $\mu$L, final pH of 7.8) or in water (15 $\mu$L, final pH of 6.0). Complexes were then incubated for 30 min with increasing charge excess of heparin (Sigma-Aldrich, Saint-Quentin-Fallavier, France). The mixture was then loaded onto a 2% agarose gel containing 1 mM EDTA and 40 mM Tris acetate buffer pH 8.0, and subjected to electrophoresis for 30 min at 90 V. After staining with ethidium bromide solution (0.5 $\mu$g/mL) for 15 min, siRNA released from polyplexes by heparin

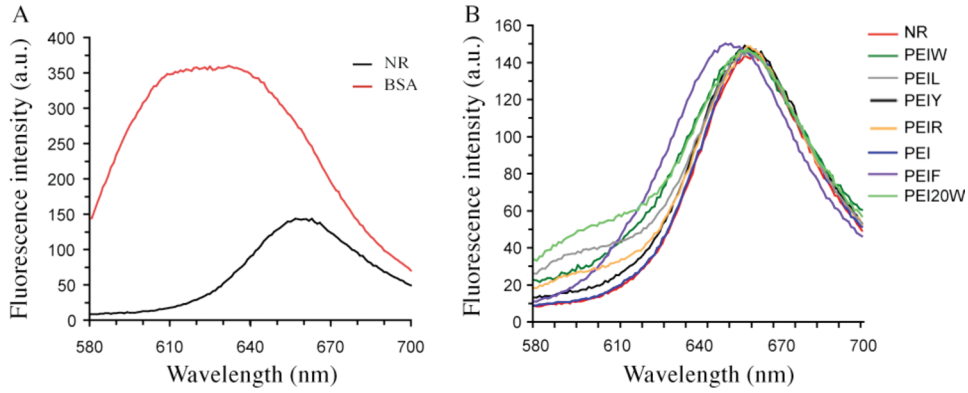


**Figure 2.** Determination of polymer hydophobicity using a fluorescent probe. Fluorescence emission spectra of Nile Red (0.56 $\mu$M) in the presence of bovine serum albumin (5 $\mu$M) (A) or with the indicated polymer (0.4 mM) (B). Excitation was at 540 nm and polymers were in RPMI medium.

was visualized with a UV transilluminator and quantified using picture analysis software (NIH *ImageJ*).

**Cell Culture.** All cell culture media were supplemented with 10% FBS (Perbio, Brebières, France), 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin (Eurobio, Courtaboeuf, France). Cells were maintained at 37 °C in a 5% $CO_2$ humidified atmosphere.

A549 cells (human lung carcinoma; CCL-185; ATCC) were transformed to stably express the *Photinus pyralis* luciferase gene originating from the pGL3 plasmid (Clontech, Mountain View, CA) and selected on G418. The transfectants A549Luc were grown in RPMI 1640 medium containing 2 mM L-glutamine (Eurobio, Courtaboeuf, France) and 0.8 mg/mL G418 (Promega, Charbonnières, France). HeLa cells (human cervix epithelial adenocarcinoma; CCL-2; ATCC) were grown in Eagle's MEM medium.

**Delivery Experiments.** The procedure is for 24-well plates or 4-well-chambered cover glass experiments. The day before experiment, cells were plated at 25 000 cells per well for A549Luc cells and 50 000 cells per well for HeLa cells. 100 $\mu$L of polyplexes, prepared as described above, were added in each well by dilution with the cell medium containing serum (0.5 mL). To ensure optimal cell growth, 24 h after polyplex addition each well was filled with an optional addition of serum-containing growth medium (0.5 mL). In the case of experiments with high concentrations of serum, growth medium was added 4 h after polyplex addition to achieve 10% serum-containing medium. The gene expression profiles were analyzed 48 h after addition of the complexes.

**Confocal Microscopy.** A549Luc cells were cultured on 4-well-chambered cover glasses (Lab-Tek, Nunc). Complexes were prepared and added to cells as described above except that siRNA and PEIY were replaced with Cy-3-siRNA and carboxyfluorescein-PEIY, respectively. After 4 h of incubation, the cell culture medium was removed and replaced with complete growth medium containing no phenol red (Sigma-Aldrich, Saint-Quentin Fallavier, France). Living cells were analyzed with a Leica TCS SP2 AOBS confocal microscope coupled to the Leica Confocal Software (Leica Microsystems), using 63× oil-immersion objective and excitation at 488 and 543 nm. Confocal sections were taken every 1 $\mu$m. Images showing one section of the middle of cells were processed by Photoshop (Adobe Systems Incorporated).

**Luciferase Profile Analysis.** Luciferase gene expression was determined 48 h after delivery with a commercial kit using manufacturer's protocol (Promega, Charbonnières, France). The luminescence was measured from 1 $\mu$L of lysate during 1 s with a luminometer (Centro LB960 XS; Berthold, Thoiry, France). Luciferase activity was expressed as light units integrated over 10 s (RLU) and normalized per milligram of cell protein by using the BCA assay (Pierce, Brebières, France). The error bars represent standard deviation derived from triplicate experiments. Luciferase silencing efficiency of pGL3luc with siRNA was calculated relative to cells treated with a nonspecific siRNA (GL2luc).

**Detection of p53 Level.** For immunohistochemistry imaging, cells were washed with PBS and fixed with 4% paraformaldehyde solution 48 h after addition of the complexes. p53 was detected with anti-p53 rabbit polyclonal serum FL-393 (Santa Cruz Biotechnology) followed by Alexa Fluor 488 labeled goat antirabbit immunoglobulins (Molecular Probes). DAPI was used for counterstaining nuclei. For Western blot analysis, cells were washed with PBS and harvested by trypsination. Each cell pellet was lysed in 50 mM Tris-HCl buffer, pH 8, containing 150 mM NaCl, 0.1% SDS, 1% NP40, 1 mM DTT, and protease inhibitors (Complete, Roche). Protein levels were next quantified using a Bradford assay and 50 $\mu$g lysate from each sample was subjected to 12% SDS−polyacrylamide gel electrophoresis and electro-transferred to nitrocellulose membrane. p53 and actin polypeptides were revealed using monoclonal anti-p53 (DO-7, Santa Cruz Biotechnology) and rabbit polyclonal anti-actin (Sigma-Aldrich) antibodies, respectively, using enhanced chemiluminescence kit (ECL kit, Millipore).

## RESULTS AND DISCUSSION

**Hydrophobic Character of the Polymers.** The hydrophobic character of each α-aa-PEI was evaluated using Nile Red (NR) as a fluorescent probe. Nile red is a zwitterionic fluorescent molecule that is sensitive to local polarity but pH-insensitive, and has been used to monitor hydrophobic surfaces of proteins (*26*). In a polar environment, Nile Red is weakly fluorescent. In a more hydrophobic environment, its quantum yield increases and its maximum emission is progressively blue-shifted. For example, interaction of Nile Red with hydrophobic surfaces of bovine serum protein (BSA) led to a 2.5-fold increase in its quantum yield as well as a remarkable blue shift of the emission maximum from 660 to 630 nm (Figure 2A). No modification of the NR emission spectrum was observed for PEI alone or for PEIs modified with the hydrosympathetic amino acids R or mixed Y (Figure 2B). The probe did not sense large hydrophobic domains on PEIL, even if this residue has a high hydropathy index, probably because the aliphatic chain is too short for formation of stable micelles/domains for NR insertion (*27*). A slight shoulder in the NR spectrum below 630 nm is seen and may be tentatively attributed to highly labile and weak interactions between the probe and solvated aliphatic chains. The impact of PEIW on the NR spectrum is a function of the modification degree. As previously seen with PEIL, interaction

**Table 1. Self-Aggregation Abilities of α-Amino Acid Modified Polyethylenimines**

| polymer | PEI | PEIR | PEIY | PEIW | PEIF | PEIL | PEIY20[a] | PEIW20[a] |
|---|---|---|---|---|---|---|---|---|
| hydropathy index[b] | | −4.5 | −1.3 | −0.9 | 2.8 | 3.8 | | |
| aggregation pH[c] | [d] | [d] | 6.3 | 5.2 | 6.9 | [d] | 7.25 | 6.4 |
| sizes of the polymer self-aggregates (nm)[e] | [d] | [d] | 325 ± 28 | 475 ± 11 | 700 ± 34 | [d] | 160 ± 40 | 218 ± 2 |

[a] Polymer with a 20% degree of modification. [b] Obtained from ref 40. [c] Corresponds to the pH for which polymer goes for a soluble to an aggregated state. The value was estimated by turbidity upon titration of a 20 mM polymer HCl solution with NaOH. [d] No DLS signal detected. [e] Estimated in RPMI media, pH 7.8, from DLS data with 120 μM of the polymer.

of NR with the indoles of PEIW20 also leads to an NR spectrum with a similar shoulder but of higher intensity, presumably for the same reason as previously indicated. No shoulder is observed with the PEIW that was modified at a 30% content, indicating that the indoles of the PEIW are no longer accessible (not solvated) for interaction with the NR probe and they probably interact together in domains without affinity for the NR. Only PEIF (hydropathy index of 2.8) induces a modest 7 nm blue shift of the NR spectrum but without enhancement of the probe quantum yield. Overall, these results indicated that modification of PEI with the selected amino acids does not lead to extensive hydrophobic domains or pockets in which Nile Red can bind. With the notable exception of aliphatic leucine, this method did nonetheless make it possible to confirm the direct transfer of the hydropathy character from the individual amino acid to the modified PEIs.

**Aggregation Properties of the Polymers.** As hydrochloride salts, all polymers are fully soluble in water even at 0.5 M concentration and can be stored in solution for extensive periods at 0−4 °C or at −20 °C. However, the amino acid modified PEIs exist in a wide variety of protonation states, which can affect their solvation in aqueous media. For example, addition of these polymers in RMPI cell growth medium, pH 7.8, provokes self-aggregation of the polymers PEIY, PEIW, PEIF, PEIY20, and PEIW20 (Table 1). In the next experiment, we determined precisely the pH value where aggregation occurs. Each polymer−HCl salt (20 mM) was carefully deprotonated with NaOH until turbidity and the pH value was measured (Table 1). PEI, the hydrophilic PEIR, and the aliphatic PEIL do not aggregate up to pH 9.0. On the other hand, the aromatic amino acid modified PEIY, PEIW, and PEIF lead to aggregation at pH of 6.3, 5.2, and 6.9, respectively, and in a narrow range (transition from a limpid to a turbid solution occurred within 0.1 pH unit). Decreasing the tyrosine or tryptophane content from 30% to 20% is conducive to aggregation at higher pH (around 1 pH unit higher) and to a softer transition. These results indicate that the aggregation abilities of the modified PEIs vary as functions of the pH and of the nature of the amino acid.

**Evaluation of the Membrane-Destabilizing Properties and Cytotoxicity of the Polymers.** Technically, nucleic acid translocation into the cell must involve a rupture of the lipid membrane integrity. Some amino acid−PEI conjugates may induce direct membrane destabilization via interfacial activity of the amino acid side chains (*28*). We therefore evaluated the effect of the polymers on mammalian plasma cell membranes by monitoring the release of hemoglobin from sheep erythrocytes (Figure 3A). PEIF is the only polymer with important hemolytic activity at 1 mM concentration. At higher concentration (10 mM), PEIL shows partial hemolysis, whereas all other PEIs have no hemolytic activity. One important property that was unique to PEIW but not to PEIW20 was that it resulted in red cells becoming brownish at pH 7.4, without apparent release of heme in solution, suggesting interference with membrane stability.

In parallel, we evaluated the toxicity level of the polymers on A459Luc cells by measuring the mitochondrial activity using the MTT assay and reported the maximum polymer concentration for which cells remained 100% viable (Figure 3B).

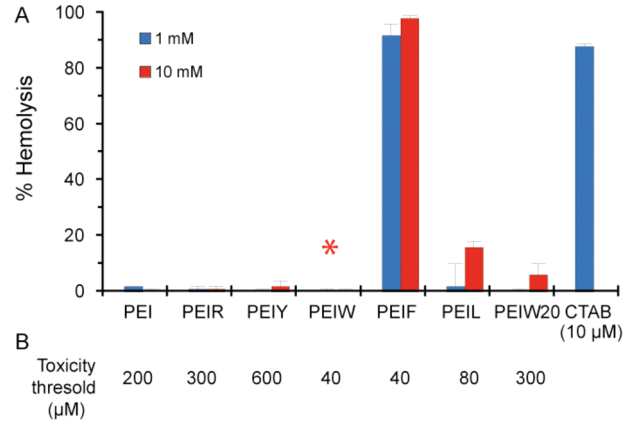


**Figure 3.** A. Permeation effect of polymers on mammalian cell membranes at extracellular pH. Sheep erythrocytes were incubated for 2 h at 37 °C with 1 and 10 mM of the various polymers in PBS, pH 7.4. Cells were then centrifuged and rupture of erythrocyte membranes was monitored by release of hemoglobin in the supernatant. Cationic detergent cetyl trimethylammonium bromide (CTAB) was used at 10 μM. [*] PEIW modifies the color of the cells from red to brown at pH 7.4 at both concentrations. B. Maximum concentration of polymer which did not provoke cell toxicity as measured by MTT assay on A549Luc cells.

Membrane-perturbing polymers PEIF, PEIL, and PEIW affect considerably the cell viability, while the others are friendlier to cells. In particular, PEIY offers an important cushion between its effective concentration (20 μM) and its toxicity threshold (600 μM). Altogether, these experiments show that PEIY does not possess any hemolytic activity and that the polymers with membrane-perturbing activity at extracellular pH rather lead to cell lethality.

**Evaluation of siRNA/PEIY Polyplexes Suitability for Entry into the Cellular Degradative Endosomal/Lysosomal Compartment.** The previous experiment showed that direct permeation of the plasma membrane is not an efficient translocation method. Translocation should rather be a consequence of a more localized rupture of lipid membranes surrounding endocytosed complexes. To take this route, most synthetic delivery systems use initial electrostatic anchorage to sulfated (polyanionic) proteoglycans present on membranes of adherent cells. While polyanions may effectively displace siRNA from polyplexes, we investigated the stability of the siRNA polyplexes in the presence of heparin, a natural polymer with high anionic density. siRNA polyplexes were prepared in RPMI medium (pH 7.8) at an N/P ratio of 50 to ensure full complexation and then incubated for 30 min with increasing quantities of heparin. The mixtures were then subjected to analysis by agarose gel electrophoresis. Release of siRNA was monitored after ethidium bromide staining and quantified (Figure 4). Heparin effectively displaces PEI from the weakly cohesive siRNA/PEI complexes and provokes a quantitative liberation of siRNA, even at a low heparin to ethylenimine ratio. According to the working hypothesis, transformation of the soluble PEI into cohesive species does indeed allow the stabilization of the polyplexes against electrostatic displacement, but to various extents. PEIY20 and PEIF, for which aggregation in water occurs at

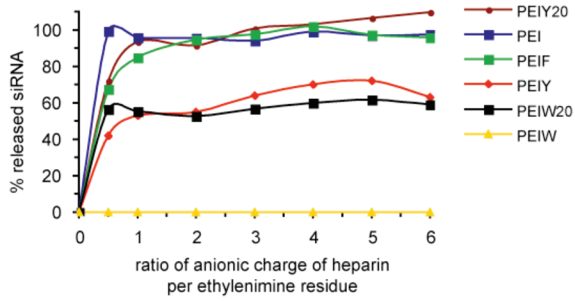


**Figure 4.** Evaluation of the electrostatic stability of the polyplexes. siRNA polyplexes (N/P 50, 0.7 $\mu$g siRNA) were incubated for 30 min with increasing amounts of heparin in RPMI medium, and release of siRNA was determined from agarose gel electrophoresis analyses.

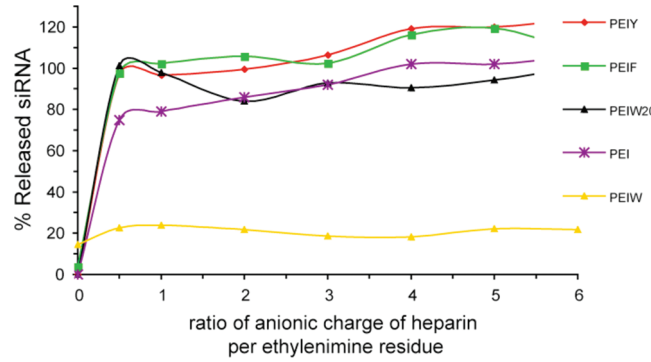


**Figure 6.** Evaluation of the electrostatic stability of the polyplexes at endosomal pH. siRNA polyplexes (N/P 50, 0.7 $\mu$g siRNA) were incubated for 30 min at pH 6.0 with increasing amounts of heparin. Release of siRNA was determined from agarose gel electrophoresis analyses.

pH 7.25 and 6.9, respectively, form moderately stable siRNA polyplexes against electrostatic displacement. On the other hand, PEIW assembles and entraps siRNA into an extremely resistant shell, as judged by the absence of siRNA liberation even at high heparin amount. An intermediate siRNA release profile is observed for the two most effective siRNA polyplexes made with PEIY or PEIW20. A fast siRNA release, obtained by displacement of superficially polyplex-bound siRNA, is followed by a plateau, for which about 40% of the RNA duplexes remained entrapped, presumably within the insoluble complex cores. These experiments suggest that the relatively low efficiency of PEIF and PEIY20 is due to instability of their self-assemblies, leading to impaired cell anchorage. Self-aggregation of PEIY, PEIW20, and PEIW is cohesive enough to protect siRNA from dissociation against electrostatic competition in an extracellular medium.

**Intracellular Fate of siRNA/PEIY Complexes.** We next followed delivery of the siRNA/PEIY complexes using Cy3-labeled fluorescent siRNA and fluorescein-labeled PEIY (green-PEIY). First, the delivery efficacy of the green-PEIY was compared to that of native PEIY using A549 cell line that stably express the pGL3 luciferase gene (A549Luc) (*22*). The polymer (12 nmol in ethylenimine residue) was mixed with siRNA (6 pmol in duplex of either targeting or untargeting sequence) in RPMI cell growth medium (100 $\mu$L) and the cationic complexes added to cells that were grown in 10% serum-containing RPMI medium. Cells were lysed 48 h later. Measured luciferase activity showed that the fluorescent tag does not significantly modify the delivery properties of PEIY (Figure 5A). Fluorescent polyplexes were then prepared and incubated under identical conditions. The fluorescent species were observed with a scanning fluorescent microscope 4 h later on living cells (Figure 5B,C). Tracking the siRNA/PEIY from the PEIY species (Figure 5B) provides images similar to those previously observed for PEI/DNA complexes (*29*). Indeed, PEIY appears as discrete spots located on the cell surfaces and in the cytoplasm in perinuclear areas, indicating that these complexes enter via the same degradative lysosomal pathway. The siRNA displays a different pattern (Figure 5C) with intracellular patches, corresponding to PEIY complexes, and a diffuse fluorescence in the cytosol. Observation of siRNA diffusion in the cytosol to that extent was quite surprising because siRNA delivered with a cationic lipid did not show such a diffuse pattern (*22*), even if the size of siRNA duplexes does not restrict cytosolic diffusion (*30*).

To explain this phenomenon, we hypothesized that acidity of the endosome may render the PEIY soluble and lose the siRNA/PEIY polyplexes. Sulfated proteoglycan receptors, which are concomitantly engulfed in endosomes, may play a favorable role here by favoring siRNA release from polyplexes. We therefore investigated once again the stability of the siRNA polyplexes in the presence of heparin but at an endosomal pH. Complexes that enter the degradative lysosomal trafficking pathway undergo progressive acidification (within 3 to 4 h) of their environments from pH 7.4 to as low as 4.5. The buffering capacity of PEI nonetheless blocks acidification of PEI-loaded endosomes at measured pHs of 5.9 (*31*) or 6.1 (*32*). siRNA polyplexes were hence incubated in the presence of increasing proportion of heparin at pH 6.0 and siRNA release reported in Figure 6. Even if low pH environments increase the cationic charge density of the PEI and strengthen the electrostatic polyplex cohesion (*33*), heparin effectively dissociates siRNA polyplexes made from the now soluble polymers PEIY and PEIW20. As previously observed at higher pH, still insoluble PEIW retains siRNA within its self-assemblies, explaining why this polymer leads to modest siRNA-mediated luciferase silencing. To complete this study, we evaluated the hemolytic activities of the polymers at pH 6.0. Results (Supporting Information, Figure S6) confirmed that PEIY has no hemolytic activity at 1 and 10 mM concentrations, suggesting that endo-

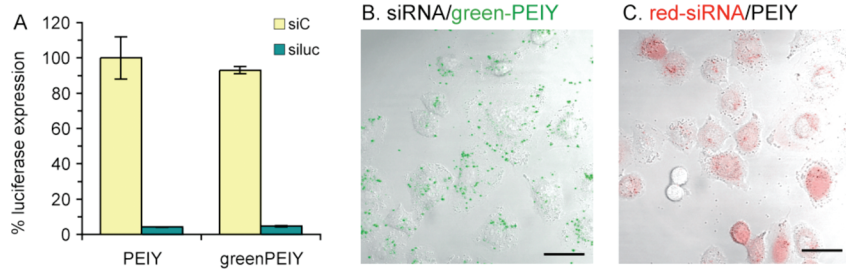


**Figure 5.** Evaluation of the PEIY delivery efficiency and intracellular fate of siRNA and PEIY following application of complexes onto cells. A. Targeting (siluc) or untargeting (siC) siRNA (10 nM) were delivered to A549Luc cells with 20 $\mu$M of PEIY or green-PEIY. Luciferase expression was measured 48 h later. For images A and B, complexes of fluorescently Cy3-labeled siRNA (red, 6 pmol) and fluorescein-labeled PEIY (green, 12 nmol) were applied to A549Luc cells grown in RPMI medium containing serum. The living cells were analyzed 4 h later by scanning fluorescence microscopy. The scale bars represent 40 $\mu$m.

somal membrane rupture for this polymer results purely from the PEI proton sponge mechanism.

Altogether, these experiments suggest that sticky PEIY and PEIW20 possess just the right hydrophobic/hydrophilic balance for carrying siRNA into endosomes and for directing siRNA release at endosomal pH values.

**Impact of Self-Aggregation on siRNA-Mediated Delivery.** Electrostatic condensation of a plasmid DNA with soluble polycations is a kinetically driven and almost irreversible process. When initial electrostatic stoichiometry between DNA phosphates and PEI is in favor of the latter, PEI molecules entangle in large excess with plasmid DNA molecules and form thermodynamically unstable cationic complexes. For the branched PEI, which is weakly protonated at pH 7.4, assembly at a PEI nitrogen amine (N) to DNA phosphate (P) ratio of 13 appeared to offer complexes with optimal gene transfection activities in vitro (*9*), presumably because this N/P ratio both permits an accurate cationic particle formation process (100% DNA condensation in cationic particles) and limits the occurrence of free PEI in solution.

The ability of PEIY to self-aggregate in extracellular medium implies that there is no free entity left in the solution. We therefore investigated the PEIY dose dependency of siRNA delivery by using three different siRNA concentrations (Figure 7). Regardless of the siRNA concentrations and of the N/P ratio, the histograms present a U-shape with an optimal gene silencing efficiency in a narrow quantity range of PEIY (7.6 to 12 nmol) for about 25 000 cells. This behavior emphasizes the importance of the dose of the active carrier for an effective endosomal rupture and subsequent siRNA activity (*34*−*37*). The U-shape is likely a combination of two opposite trends. A gradual decrease in luciferase activity with increasing PEIY quantity presumably corresponds to progressive titration of the particle's cell surface receptors up to full occupancy. The subsequent increase in PEIY to siRNA ratio in turn dilutes the siRNA within PEIY containing particles and diminishes the quantity of delivered siRNA into the cytosol. Decreased luciferase silencing activity is then observed and is amplified at low siRNA inputs. Although the mechanism of siRNA-mediated silencing also depends on the siRNA concentration, it is worth mentioning that 75% silencing efficiency was obtained with extracellular siRNA duplex concentration as low as 0.1 nM, reflecting nonetheless the effectiveness of such a mechanism.

**PEIY versus a Cationic Lipid Formulation for siRNA-Mediated Silencing of a Viral Oncoprotein.** To evaluate the potential of PEIY as a general siRNA delivery reagent, we next compare it to HiPerfect, a commercially available siRNA-optimized, "second-generation", cationic lipid formulation for the delivery of oncoprotein-targeting siRNAs. A majority of cervical cancers is caused by high-risk types of human papillomaviruses (HPV) and derives from genetic integration of viral E6 and E7 oncoproteins into infected cells. These two oncoproteins disrupt several cellular growth control mechanisms. Malignancy due to E6 is attributed to its ability to induce proteasomal degradation of the p53 tumor suppressor protein. The cervical cancer cells HeLa harbor copies of the HPV-18 genome, and in vitro studies showed that p53 levels can be restored by silencing E6 expression with an E6-selective siRNA (siE6) (*38*). siE6/PEIY complexes were prepared as described earlier, and the cationic lipid formulation was used according to the manufacturer's instructions. Complexes were simply added to the cells grown in complete MEM medium. The p53 level was monitored by immunohistochemistry and Western blot analysis (Figure 8). A siE6-mediated increase of nuclear p53 is observed in almost all cell nuclei to the same extent as the lipid formulation, although nonspecific antibody binding to PEIY aggregates is also seen (Figure 8A). Quantification of p53 level in the immunoblot after delivery with an untargeted siRNA (siC) or without carrier (Figure 8B) confirms that PEIY does not interfere with the cellular cascade leading to the p53 increase. These results suggest that PEIY represents an alternative siRNA delivery reagent to the lipidic ones and can be applied for silencing malignant gene expression.

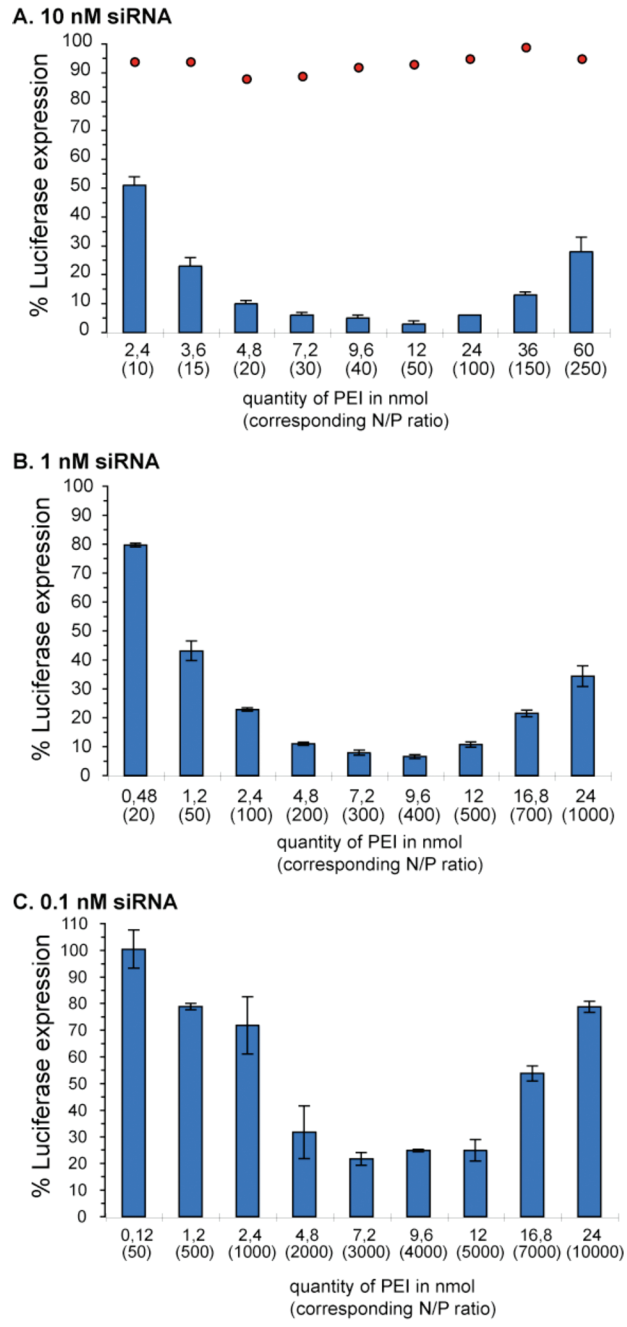


**Figure 7.** Evaluation of the siRNA delivery efficacy as a function of the PEIY quantity. The polymer at the indicated quantity was mixed with siRNA (6 pmol (A), 0.6 pmol (B), or 0.06 pmol (C)) in 0.1 mL RPMI and added to A549Luc cells grown in medium containing serum (0.5 mL). Luciferase expression was measured 48 h after addition of polyplexes. The bar represents the residual luciferase activity. The red points measured the percentage of cell protein levels relative to mock (same scale as luciferase expression).

**Stability of siRNA/PEIY Complexes in High Serum Content.** In this final experiment, we examined the stability of the siRNA/PEIY complexes in the presence of varying amounts of serum from luciferase silencing experiments.

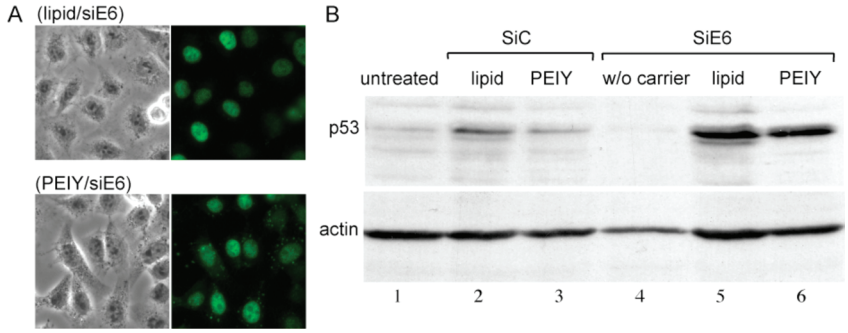


**Figure 8.** Inhibition of the HPV18 E6 oncoprotein in HeLa cells using siRNA (6 pmol) delivered with either PEIY (12 nmol) or a commercial cationic lipid (HiPerfect). The resulting increase of p53 was visualized by immunohistochemistry (A) and Western blotting (B). SiC: untargeted siRNA. siE6: E6 targeted siRNA.

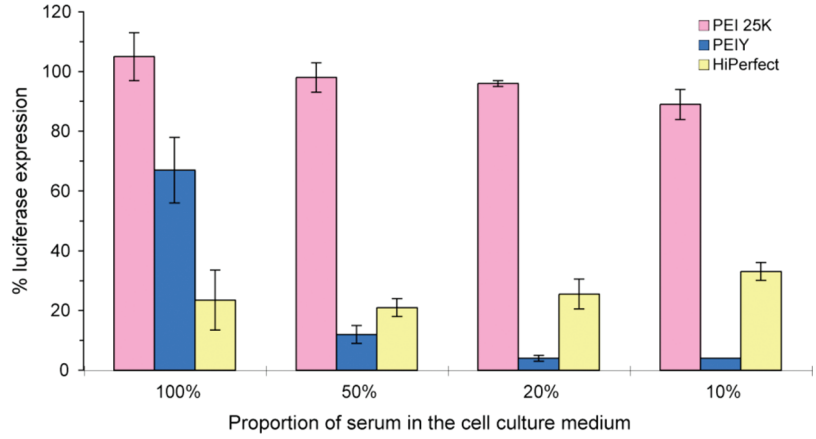


**Figure 9.** Evaluation of the PEIY delivery efficacy in the presence of high serum concentration. siRNA (10 nM) were delivered to A549Luc cells with PEI 25K (pink bars), PEIY (blue bars), or HiPerfect (yellow bars) in the presence of 100%, 50%, and 20% serum, respectively. After 4 h, the medium was replaced with the optimal cell growth medium (RPMI containing 10% serum) and luciferase expression was measured 48 h later. The results are relative to cells treated with nonspecific siRNA.

Indeed, extracellular serum contains many amphiphiles with hydrophobic surfaces, such as seric albumin, that can potentially interfere with the tyrosine-tyrosine interactions and hence diminish the cohesion of the polyplexes. Figure 9 shows that PEIY remains a remarkable siRNA delivery agent up to a 50% serum content (90% luciferase inhibition) but loses its efficiency in 100% serum. By comparison, HiPerfect-based lipoplexes appear less effective in delivering siRNA to A549 cells than to Hela cells but are fully insensitive to serum proportion in the cell culture media. These results, in combination with the absence of hemolytic activity, suggest that PEIY may be suitable for in vivo administration but is not tailored for intravenous delivery.

## CONCLUSION

Our results show that tyrosine−polyethylenimine conjugate has a favorable efficiency/toxicity balance and is able to convey effectively low quantities of siRNA, even in the presence of high serum content. The success of tyrosine−polyethylenimine does not derive from membrane-perturbing activity but may originate from a disassembly sensitivity at pH 6.0, a pH value that is found in PEI-buffered endosomes. This property emanates from the PEI proton sponge ability and favors siRNA release from polyplexes at moments when PEI conducts osmotic pressure increase on endosomal membranes. Subsequent siRNA diffusion into the cytosol may hence not be restricted when endosome swelling/lysis events take place. Regardless of its mechanism, ease of preparation at low cost and high delivery efficacies should be appreciated for gene silencing experiments in cell culture. More experiments are currently underway to address usefulness of such modified PEIs in vivo for administration and pathologies for which cell surfaces are accessible to large delivery systems (*36, 39*).

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**Supporting Information Available:** Detailed discussion on the synthetic design of the polymers, procedures for polymer synthesis and toxicity assay. Figure S1 shows NR spectra in presence of the various polymers in 150 mM NaCl. Figures S2−3 are the cell viability dose response to the polymers. Figure S4 shows the efficiency of PEIY to deliver siRNA following siRNA/PEIY polyplexes assembly in various media. Figure S5 shows confocal image analysis of A549Luc cells that were treated with Cy3-siRNA and its corresponding PEI polyplexes. Figure S6 is the hemolysis assay at pH 6.0. Figures S7 and S8 are the agarose gels used to plot the Figures 4 and 6. Figure S9 shows siRNA-mediated cyclinD1 silencing using PEIY as the delivery carrier. This material is available free of charge via the Internet at http://pubs.acs.org.

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