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Self-Assembling Polyethylenimine Derivatives Mediate Efficient siRNA Delivery in Mammalian Cells

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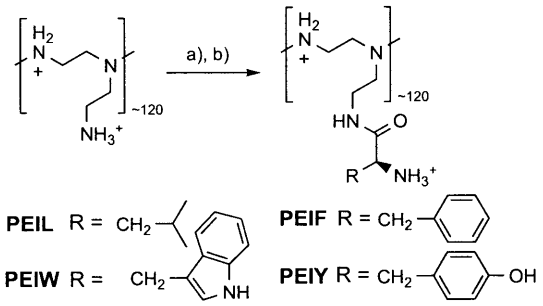
Synthetic 21–22-nt-long RNA duplexes (siRNAs) can trigger specific mRNA degradation and selectively control gene expression, and because of this they have potential therapeutic applications.[1] Unfortunately, the intracellular localization of siRNA targets and the inability of oligonucleotides to diffuse across cellular membranes requires siRNA conjugation or formulation with synthetic delivery vectors.[2] Interestingly, polyethylenimine (PEI), a popular gene transfection agent[3] that showed promise for cancer gene therapy,[4] appears inefficient for siRNA delivery in vitro.[5]

The poor performance of PEI could be linked with the length of the siRNA anionic segment, which is too short to maintain electrostatic cohesion with the soluble cationic polymer. Consequently, siRNA polyplexes break apart too readily upon contact with polyanions present at cell surfaces.[6] We reasoned that the transformation of water-soluble PEI into a molecule with self-assembly properties should stabilize siRNA polyplexes and hence favor overall siRNA delivery.

A water-soluble polymer could acquire new aggregating properties through the addition of hydrophobic domains. We avoided using alkyl chains, which, by mixing with the cell lipid bilayer, might act as a detergent and cause cell death. Our preference was instead for natural α-amino acids with decreasing hydropathy indices,[7] such as leucine (L), phenylalanine (F), tryptophan (W), and tyrosine (Y). Indeed, these amino acids constitute the cores of globular proteins and help maintain protein structures. They also can be coupled to PEI amines without destroying nucleic acid binding properties, because over the course of the reaction a cationic amine replaces the reacted one.

Commercially available 25 kDa branched PEI contains primary, secondary, and tertiary amines in a ratio of 1:1:1.[8] The primary amines were allowed to react fully with the succinimidyl esters of butyloxycarbonyl-protected amino-acids (Boc-aa-OSu) (Scheme 1). Removal of the Boc groups with trifluoroacetic acid (TFA) and subsequent dialysis in aqueous HCl gave the desired products, with α-amino acid contents of 30% per ethylenimine, in 30–60% overall yields.

The aggregating properties of the polymers were evaluated by measurement of dynamic light scattering (Table 1). All polymers, as hydrochloride salts, were soluble in water, even at a concentration of 0.5 M. Upon dilution in RPMI cell culture medium, no light scattering signals were detected in the cases of PEI and PEIL. On the other hand, PEIF, PEIW, and PEIY self-assembled into particles with apparent diameters between 0.3–0.7 μm. Addition of siRNA permitted PEI and PEIL to form complexes and led to slight increases in the sizes of the self-assembling polymers in a range that had previously been observed to be effective for PEI-mediated gene delivery.[9] Transmission electron micrographs showed that PEIY (Figure 1A) forms fiber-like structures of fused irregular spheroids roughly 20 nm in diameter. Addition of siRNA (Figure 1B) led to a twofold diminution in the fiber diameters and to further clustering.



Scheme 1. Synthesis. a) Boc-aa-OSu. b) TFA.

Table 1. Sizes [nm] of polymer self-assemblies in RPMI medium.

Polymer[a]	Alone	+ siRNA[b]
PEI	–	155 ± 31 nm
PEIL	–	175 ± 13 nm
PEIF	700 ± 34 nm	880 ± 66 nm
PEIW	475 ± 11 nm	620 ± 41 nm
PEIY	325 ± 28 nm	570 ± 33 nm

[a] 120 μM in ethylenimine. [b] 2.4 μM in phosphate.

Nanoparticles, including viruses, are too large to diffuse freely across membranes and thus need to divert the cell machinery for translocation. They first anchor to receptors on the cellular surface and are then actively dragged into cells within membrane-coated vesicles (often endosomes).[10] Evidence suggests that the high buffer capacity of PEI enables rupture of the endosome membranes and hence effective nucleic acid translocation.[11] We therefore examined whether amino acid modification impacted this important function by acid–base titration (Figure 1). Profiles of modified PEIs superposed almost perfectly in the pH 6.0–8.0 range and showed that the polymers have higher buffering capacities than PEI, probably due to the electron-withdrawing effect of carboxamide on the α-terminal amines. This greater "proton sponge" ability should

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: protocols for polymer synthesis and delivery experiments.

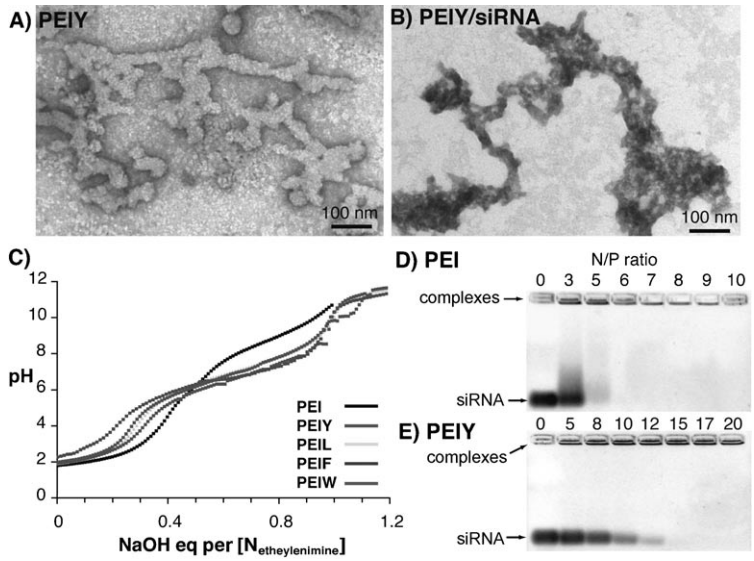

Figure 1. TEM pictures of PEIY, A) alone, or B) with siRNA (see Table 1 for conditions). C) Titration profile of modified polymers showed an increased buffer capacity relative to PEI. Gel retardation assays showed complexation of siRNA by PEIY (gel E) and PEI (gel D).

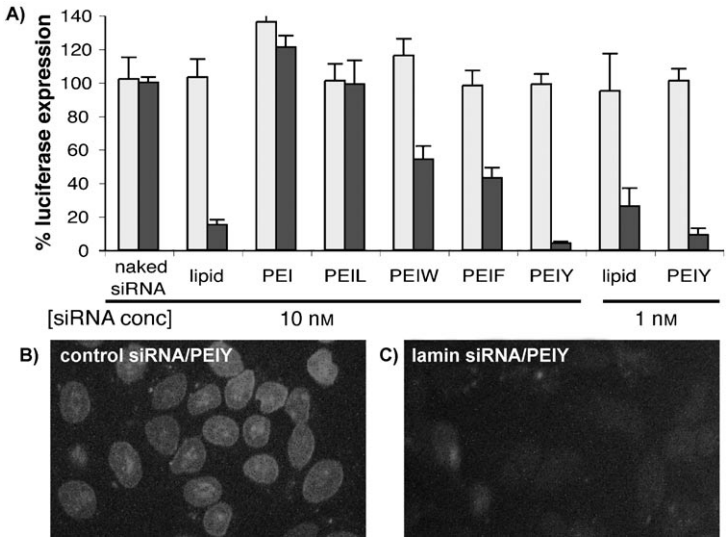

Figure 2. A) The efficiency of the polymers in delivering siRNA was monitored by measuring the luciferase activity with control siRNA (light bars) and luciferase siRNA (dark bars). B), C) Silencing of lamin A/C in HeLa cells with 10 nM of B) control siRNA, or C) lamin siRNA delivered with PEIY. Lamins appear in green after immunostaining.

ensure more proton capture during endosome acidification and therefore better delivery.[12] However, this property results in fewer ammonium ions, which may weaken siRNA binding. At pH 8.0, only 18% of the amino nitrogen atoms of the modified polymers are still protonated, as compared to 40% for PEI. Nevertheless, they were still able to bind siRNA and to form electrophoretically stable siRNA polyplexes, and as expected, the PEIY ratios of ethylenimine nitrogens (N) to nucleic acid phosphates (P) were roughly twice that of PEI (Figures 1D, 1E).

The abilities of the polymers to convey siRNA were evaluated through the use of the firefly pGL3 luciferase gene as the targeted reporter. To avoid experimental bias generated by transient plasmid transfection, we used A549 cells, which stably express the pGL3 gene. One of the most efficient routes for entry of synthetic delivery systems into endosomes uses initial electrostatic anchorage to sulfated proteoglycans present on cell membranes.[10] One necessary condition for its employment is the formation of cationic particles.[13] Particles were thus prepared by mixing an excess of the polymer (12 nmol in ethylenimine) with siRNA (6 pmol) in RPMI (100 μL). The resulting cationic assemblies (ζ of $+20$ mV) were then simply added to cells (grown in the presence of serum) to reach a final siRNA concentration of 10 nM. Cells were lysed 48 h later, and the efficacies of the polymers were compared to that of Interferin, a commercially available lipid optimized for siRNA delivery (Figure 2A). Water-soluble PEI and PEIL were inefficient, whereas aggregating PEIW, PEIF, and PEIY produced siRNA-mediated inhibition of luciferase expression. The PEIY was particularly impressive, even at doses as low as 1 nM. (Silencing of 96 and 91% was observed for 10 and 1 nM, respectively). The vector altered neither the cell viability (for details see the Supporting Information), nor the siRNA selectivity, because untargeted siRNA did not inhibit luciferase production. PEIY was also effective in delivering another siRNA in a different cell line, as exemplified by knockdown of lamin A/C expression in HeLa cells (Figures 2B, 2C). The efficacy of PEIY seems to be mainly related to its hydrophobic/hydrophilic balance and was decreased upon decreasing the tyrosine to 20%. The opposite trend was observed for the more hydrophobic PEIW, which achieved 90% gene silencing at a lower tryptophan content (Supporting Information).

In summary, we have shown that simple but potent siRNA delivery molecules can be rationally conceived. Work to evaluate these delivery vehicles in vivo is in progress.

Experimental Section

Protocols for polymer synthesis and for delivery experiments are described in the Supporting Information.

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