siRNA Delivery

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Polyspermine Imidazole-4,5-imine, a Chemically Dynamic and Biologically Responsive Carrier System for Intracellular Delivery of siRNA**

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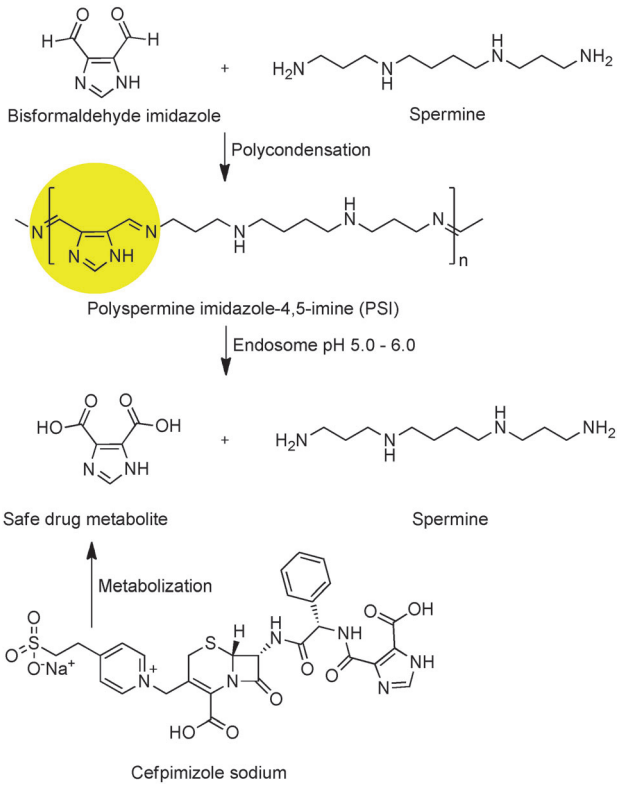
The ability of small interfering RNA (siRNA), a polynucleotide approximately 21–23 nucleotides in length, to inhibit the expression of target genes has offered scientists new methods to develop customizable medicines.[1] Recent advances in siRNA research have resulted in solutions to multi-target and off-target problems of siRNA.[2] The lack of a safe and efficient synthetic carrier system has become the sole remaining barrier to bringing this revolutionary finding to practical therapeutic technology.[3] A therapeutically feasible synthetic carrier of siRNA should be able to accomplish these five sequential tasks: A) packing siRNA tightly into nanoparticulate forms to avoid degradation prior to phagocytosis, B) adsorbing selectively onto diseased cells, C) rupturing the endosomes to facilitate endosomal escape of siRNA, D) releasing siRNA into the cytoplasm, and E) becoming metabolized into nontoxic and eliminable species.[4] Among these five tasks, (A), (C), (D), and (E) may be achieved with a rationally designed polycationic carrier system, because these four steps are universal for all cell types. Herein, we report a multifunctional, yet structurally simple, cationic polymer, polyspermine imidazole-4,5-imine (PSI), which accomplishes these four tasks.

This polymer is formed by condensing spermine, an endogenous molecule whose role is the packaging of genes in sperm, with bisformaldehyde imidazole, an intermediate which may easily be oxidized to a metabolite which is known to be safe,[5] through a pH-responsive linkage, a bisimine bond conjugated with the imidazole ring. Our hypothesis is that the imidazole-conjugated π linkage (Scheme 1) has a pK_a in the range of 5–6, which causes it to be stable at neutral or basic pH values, but dissociate to safe metabolites within the acidic conditions of the endosome. Moreover, each of the repeating units of the polymer



Scheme 1. Synthesis, chemical structure, and pH-responsive metabolism of polyspermine imidazole-4,5-imine (PSI).

generates a spermine, with two primary amino groups upon degradation. These free amino groups absorb two additional protons, further propagating the proton-sponge effect,[6] and in turn facilitating endosomal escape of siRNA.[7] When polymer degradation occurs, the packed siRNA is released, and the polymeric carrier itself is metabolized to spermine, an endogenous molecule, and imidazole bisformate. Since the endosome may be reformed before the engulfed particle escapes,[8] the concurrent proton absorbtion and particle dissociation of the PSI/siRNA polyplex may offer an additional advantage by avoiding re-encapsulation by the endosome.

For intercellular targeting of diseased cells (task B), a carrier system must be assembled in a way in which selected cell-targeting moieties can be easily and firmly attached to the surface of the polyplex with enough flexibility to optimize the population. These criteria can be met through the assembly of a functional monolayer, comprised of an amphiphilic block copolymer surrounding the polyplexes[9] (this study to be published elsewhere).

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The synthesis of PSI was as simple as a single condensation reaction (Scheme 1): adding an approximately equivalent molar amount of imidazole-4,5-dialdehyde to spermine (both dissolved in dimethyl formamide (DMF)) in the presence of a catalytic amount of *p*-toluenesulfonic acid lead to the formation of PSI. The polymerization was allowed to proceed at 80 °C for 24 h, with stirring, followed by filtration, evaporation, and dialysis in ultrapure water through a cellulose membrane (10 kDa molecular weight cut off). The final product was lyophilized to give a yellowish powder, prior to storage at −80 °C.

The identity and average molecular weight of the synthesized PSI were confirmed by various methods, including ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, FTIR, and size exclusion chromatography (SEC; see the Supporting Information, Figure S1). As shown in Figure S1, the disappearance of proton signal at $\delta = 10.1$ ppm, and the generation of one at $\delta = 7.5$ ppm in the ^{1}H NMR spectra indicated conversion of bisaldehyde (CH=O) into bisimine (C=NH) during the reaction. These results were corroborated by the disappearance of a carbon signal at $\delta = 184.9$ ppm and the appearance of one at $\delta = 163.2$ ppm. Additionally, the intense absorption at 1647.4 cm^{-1} in the FTIR spectrum was attributed to the stretching vibration of the newly formed bisimine from bisaldehyde, whose stretching frequency is 1668.4 cm^{-1}. Moreover, the average molecular weight (MW) of PSI was determined to be 21.9 kDa (with polydispersity of 1.44) by eluting a solution of the polymer (2 mg mL^{-1} in water) through an SEC column (PL aquagel-OH), using a series of polyethylene glycol (PEG) standards and 25 kDa polyethylenimine (PEI) for calibration.

The ability of polycationic PSI to package polyanionic siRNA into polyplex nanoparticles was examined as a function of the ratio of amino groups of the polymer to phosphates of the nucleic acid (N/P ratio) using gel electrophoresis, atomic force microscopy (AFM), dynamic light scattering (DLS), and zeta potential measurement. As shown in Figure 1 a, the addition of PSI to the aqueous solution of siRNA resulted in retarded of electrophoretic mobility of the siRNA when the N/P ratio was over 20, indicating that the polymer was able to condense the siRNA into polyplexes. AFM images, although under dehydrated conditions, showed formation of the PSI-siRNA polyplexes, spherical in shape and 240–250 nm in average diameter (Figure 1 b). Mean particle size and the zeta potential of the polyplexes in water were determined by DLS and plotted versus N/P ratio (Figure S2). The particle sizes varied between 200–300 nm (Figure 1 c), consistent with the measurement by AFM, and the zeta potential was in the range between 10.96–15.72 mV (Figure S2). The relatively low zeta potential, as compared with many reported cationic polymer-based polyplexes, suggests that a considerable fraction of the amino groups of PSI were not protonated. These characterizations based on various methods confirmed the capability of PSI to package siRNA into polyplexes.

One of the unique characteristics of PSI is that it can degrade to spermine, with two free amino groups (for enhanced proton-sponging effect) and a safe drug metabolite, at endosomal pH values. This degradability was examined by

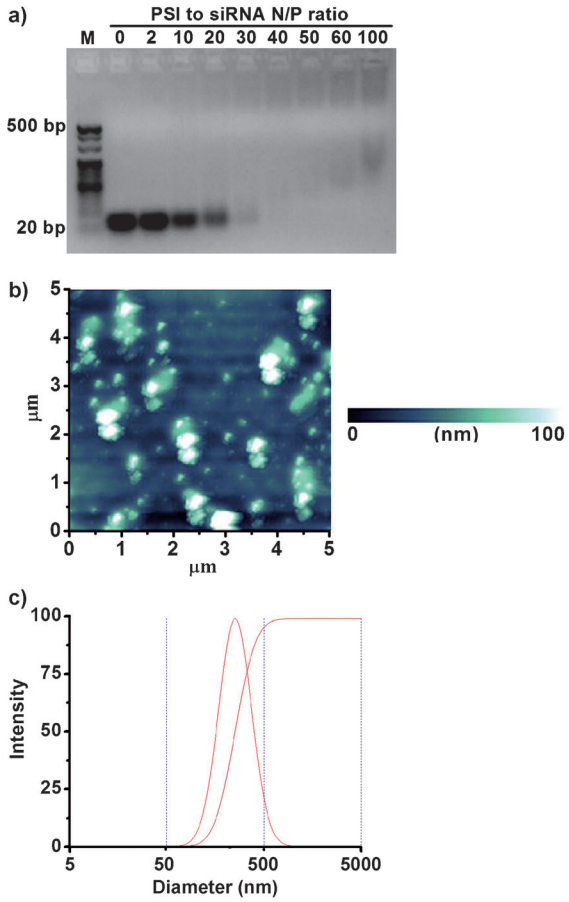

Figure 1. Physicochemical characterization of polyplexes formed by PSI and siRNA. a) Agarose gel electrophoresis of polyplexes formed from PSI and siRNA (0.375 μM) at various N/P ratios (0–100) in RNase-free water (M = 20 bp DNA ladder marker). b) Atomic force microscopy (AFM) phase image of polyplexes at an N/P ratio of 20 under dehydrated conditions. c) Particle size distribution of polyplexes at an N/P ratio of 20 in RNase-free water.

incubating the polymer in formic acid buffers, pH value 7.4, 5.8, and 5.0 at 37 °C, followed by monitoring average molecular weight (MW) changes using SEC (with 25 kDa PEI as a standard). These three pH values were selected for simulating the environment of blood plasma, endosomes, and lysosomes, respectively.

As shown in Figure 2 a, the average molecular weight of PSI declined over time at remarkably different rates in the different buffers (with pH 7.4 ≪ pH 5.8 ≪ pH 5.0). The degradation rates were determined as the time required for the average MW to reach half of its original value. This time period was 48.8 h, 22.6 h, and 0.6 h for PSI incubated in pH 7.4, 5.8, and 5.0 buffers, respectively (Figure S3). In addition, the final degradation products of PSI were determined to be spermine and imidazole biscarboxylic acid, rather than imidazole bisaldehyde (Figure S4).

A major benefit of this pH-responsive degradation of PSI is that it is non-toxic to cells. Cell viability assays were performed by culturing COS-7 cells and SMMC-7721 cells (stably transfected with the luciferase gene) with PSI, as displayed in Figure 2 b, the viability of both cell lines remains unchanged as the polymer concentration was increased to

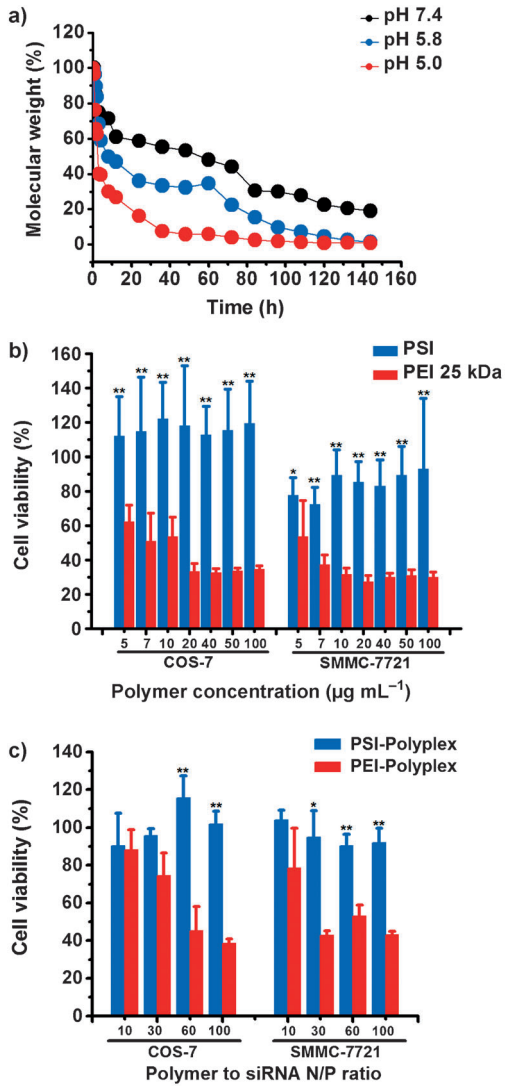

Figure 2. pH-responsive degradation and cytotoxicity of PSI. a) PSI was dissolved in formic acid buffers at pH 7.4, 5.8, and 5.0 to a concentration of 2.0 mg mL^{-1} and incubated at 37 °C, followed by SEC measurements. The molecular weight of the polymers were determined as the weight measured by SEC relative to the original molecular weight of the polymer (100 %). Viability of COS-7 cells and SMMC-7721 cells (stably expressing the GL3 luciferase gene), treated with b) PSI and 25 kDa PEI at various polymer concentrations (5–100 μg mL^{-1}) or c) polyplexes formed between PSI or 25 kDa PEI and siRNA (22.5 nM) at various N/P ratios (10–100); * = $p < 0.05$ and ** = $p < 0.01$ versus 25 kDa PEI; $n = 6$ per group; data are means ± s.d.

100 μg mL^{-1}. For the same cell lines treated with 25 kDa PEI, viability dropped dramatically to 20 % of its original value when the polymer concentration reached 20 μg mL^{-1}. A parallel assay, treating the cell lines with siRNA polyplexes formed from the two polymers at various N/P ratios (equivalent to the amount of polymers discussed above) showed the same trend as above (compare Figure 2b,c). The nontoxic effects of PSI and the PSI-polyplex were confirmed by direct observation of cell growth under a microscope to rule out possible interference of the polymer on the MTT assay of cell viability (Figure S5). Cytotoxicity is a major concern for the therapeutic use of a synthetic nucleic acid carrier.

The efficiency of PSI to deliver siRNA into cells was compared with that of 25 kDa PEI, by delivering siRNAs that silence a luciferase plasmid (pGL3) in COS-7 cells and the stably transfected luciferase gene in SMMC-7721 cells. COS-7 cells were transfected with pGL3 plasmid, which was delivered by Lipofectamine 2000, or with polyplexes with PSI or 25 kDa PEI, at each N/P ratio. This experiment confirmed that PSI and 25 kDa PEI did not affect gene expression differently (Figure 3a).

When antisense and nonsense siRNA polyplexes with PSI and 25 kDa PEI at various N/P ratios were added to the culture of COS-7 cells pre-transfected with pGL3/Lipofectamine 2000 lipoplexes, PSI polyplexes showed significantly better luciferase inhibition and higher cell viability than 25 kDa PEI at each N/P ratio. This result indicates that the reduced luciferase expression was not due to cell death (Figure 3b). For the cells treated with PSI-delivered antisense siRNA, the rate of pGL3 silencing was 70.0 %, 69.9 %, and 95.5 % (defined by the difference in luciferase expression between cells treated with antisense and nonsense siRNA) for N/P ratio of 20:1, 30:1 and 50:1, respectively. For the cells treated with 25 kDa PEI-delivered siRNA, the pGL3 silencing was 56.0 %, 58.3 %, and 30.4 % for same N/P ratios (Figure 3b). This result strongly suggests that PSI is more efficient than 25 kDa PEI in delivering siRNA to the cytoplasm, the site of gene silencing. The superior silencing efficiency of PSI supports our hypothesis that the pH-responsive degradation of the polymer facilitates endosomal escape (by releasing spermine with free amino groups), releasing the siRNA into the cytoplasm.

To examine the efficiency of PSI to deliver siRNA under in vivo conditions, fetal bovine serum (FBS) was added to the cell culture medium of SMMC-7721 cells to determine how FBS may affect gene silencing. Anti-pGL3 siRNA formulated with PSI, 25 kDa PEI (both at N/P ratio of 50/1), and Lipofectamine 2000 were added to SMMC-7721 cells in the culture media containing 0 % or 10 % FBS. The ratio of Lipofectamine 2000 to siRNA used was as suggested by the manufacturer. As indicated in Figure 3c, the addition of 10 % FBS in the cell culture compromised siRNA silencing efficiency for both polymeric carriers, but PSI was still superior to 25 kDa PEI in terms of cell viability and pGL3 suppression (Figure 3c). Nevertheless, PSI-packaged siRNA showed some activity in silencing genes in the presence of FBS.

Finally, the in vivo efficacy of PSI in delivering siRNA was examined using a nude mice model, inoculated under the skin with tumor-forming SMMC-7721 cells, stably expressing the GL3 luciferase gene. The mice received intratumor injections of PSI/antisense-siRNA polyplexes, at an N/P ratio of 50, or naked antisense siRNA or saline as a control. Luciferase expression was measured after dissecting and homogenizing the tumor tissue. As shown in Figure 3d, antisense siRNA delivered by PSI resulted in a significantly higher silencing efficiency (suppressed GL3 expression by 60–70 %) than naked antisense siRNA and saline (these two were essentially the same).

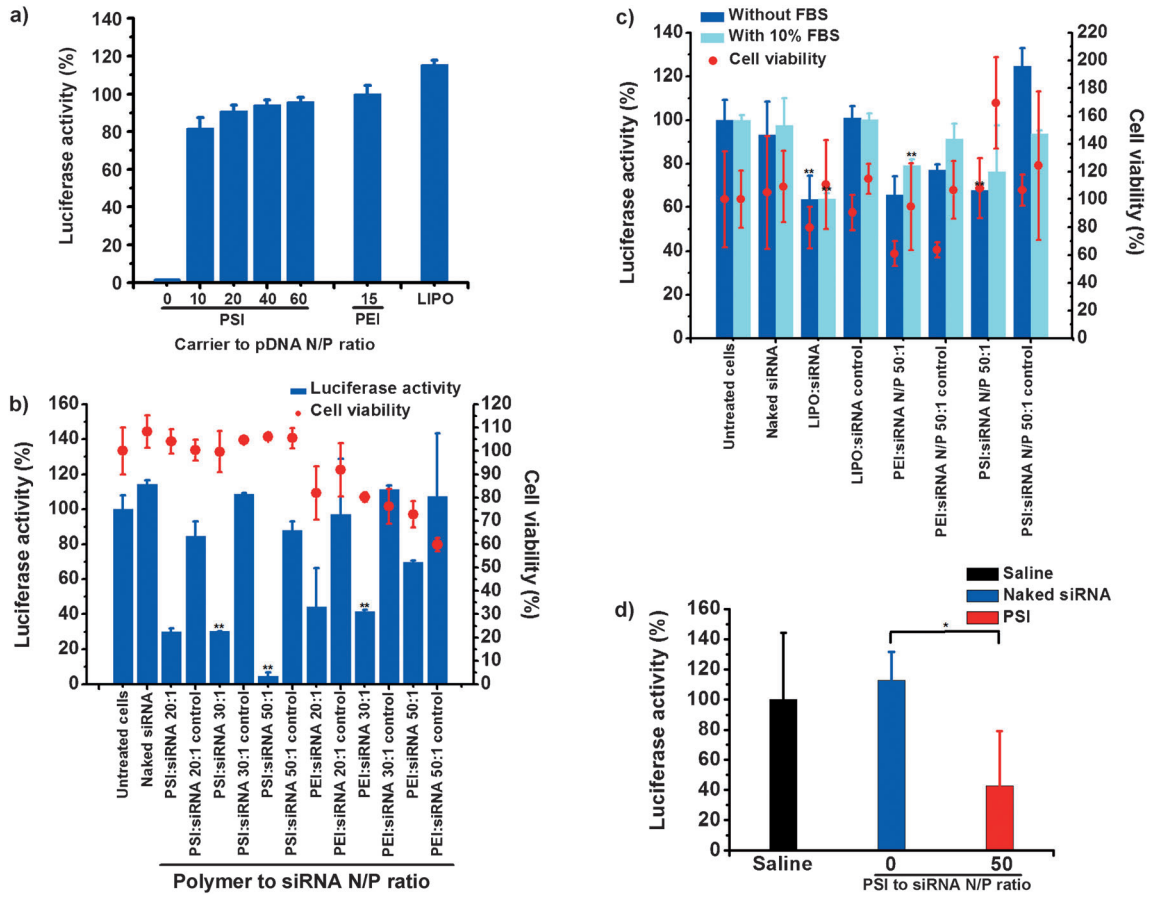

Figure 3. a) Transfection efficiency of luciferase gene plasmid (pGL3, 0.3 nM) delivered by PSI, 25 kDa PEI, or Lipofectmine 2000 (LIPO) in COS-7 cells. b) Cell viability and pGL3 silencing efficiency of siRNA (22.5 nM) delivered by PSI or 25 kDa PEI at various N/P ratios to COS-7 cells; control = scrambled siRNA control sequence. c) Cell viability and pGL3 silencing efficiency of siRNA (22.5 nM) delivered by Lipofectmine 2000, 25 kDa PEI, or PSI at various N/P ratios to SMMC-7721 cells cultured with 0% or 10% FBS; * = $p < 0.05$ and ** = $p < 0.01$ versus siRNA control. d) pGL3 silencing efficiency in nude mice of siRNA delivered in naked form or with the PSI-packaged form (N/P ratio 50) to tumor tissues; * = $p < 0.05$ versus naked siRNA mice; $n = 6$ per group; data are means ± s.d.

The experimental results discussed above demonstrated the feasibility of siRNA delivery with a rationally designed synthetic carrier. Polyspermine imidazole-4,5-imine (PSI) offers an example of a cationic polymer capable of condensing siRNA into a polyplex, releasing the siRNA from the endosome into the cytoplasm, and degrading into nontoxic components (tasks A, C, D, E mentioned above). Although the PSI polyplex alone is incapable of overcoming the difficulties of in vivo circulation and cell-specific targeting (task B) because of its positive charge and lack of targeting moieties, a rationally designed universal surface assembly is being examined in our lab to improve the serum stability of siRNA polyplexes, neutralize the cationic charges of the polyplexes, and immobilize selected cell-targeting molecules on PSI-polyplexes.

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