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Pyridylthiourea-grafted polyethylenimine offers an effective assistance to siRNA-mediated gene silencing *in vitro* and *in vivo*

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ABSTRACT

Success of synthetic interfering nucleic acids (siRNAs)-based therapy relies almost exclusively on effective, safe and preferably nanometric delivery systems which can be easily prepared, even at high concentrations. We prepared by chemical synthesis various self-assembling polymers to entrap siRNAs into stable polyplexes outside cells but with a disassembly potential upon sensing endosomal acidity. Our results revealed that pyridylthiourea-grafted polyethylenimine (πPEI) followed the above-mentioned principles. It led to above 90% siRNA-mediated gene silencing *in vitro* on U87 cells at 10 nM siRNA concentration and did not have a hemolytic activity. Assembly of siRNA/πPEI at high concentration was then studied and 4.5% glucose solution, pH 6.0, yielded stable colloidal solutions with sizes slightly below 100 nm for several hours. A single injection of these concentrated siRNA polyplexes into luciferase-expressing human glioblastoma tumors, which were subcutaneously xenografted into nude mice, led to a significant 30% siRNA-mediated luciferase gene silencing 4 days post-injection. Our results altogether substantiate the potential of self-assembling cationic polymers with a pH-sensitive disassembly switch for siRNA delivery *in vitro* and also *in vivo* experiments.

1. Introduction

Small interfering RNA duplex (siRNA) generates sequence-selective mRNA degradation, resulting in an effective silencing of the targeted gene [1]. This gene-silencing mechanism is of great value for analyzing the function of a single gene in cultured cells and holds great potential as gene-specific therapeutics [2]. High pressure tail vein injection of siRNAs (hydrodynamic delivery method) enables translocation of nucleic acids into the liver cells and provided the first demonstrations of the therapeutic potential of siRNAs [3,4]. Unfortunately, this method is too risky to be used on humans, especially on unhealthy individuals. Further progress has been achieved by formulating siRNAs within lipidic vehicles either for delivery to the liver [5] or to lung cancer cells [6]. Cationic polymers have also been used for siRNA complexation and *in vivo* delivery. Promising results were obtained for tumor therapy either using simple cationic systems [7–9], PEG-protected [10] or targeted multifunctional ones [11,12].

The polyethylenimine (PEI) is one of the most studied cationic DNA transfection reagents [13] and has been evaluated as well for siRNA delivery [14]. Encouraging siRNA-mediated gene silencing results were obtained *in vivo* with low molecular weight branched PEI [8,15] despite the weak cohesion of siRNA/PEI polyplexes in presence of cell surfaces [16]. At an *in vitro* cellular level, successful siRNA delivery with PEI showed to rely on high siRNA dose, on a branched polymer and on specific polyplex formation conditions [17]. Strategies to increase the stability of the siRNA polyplexes were developed [18]. One is to modify the polycation backbone with hydrophobic elements [9,19]. In this vein, we recently reported that modification of the "proton-sponge" polyethylenimine (PEI) 25 kDa with tyrosine led to a self-assembling polymer (PEIY) with effective siRNA delivery abilities at a cellular level [20]. Besides providing increased extracellular stability, non-covalent interactions have the advantage of potential reversibility. A further detailed investigation indicated that mildly acidic conditions (pH 6.0), such as ones encountered in PEI-buffered endosomes [21], weaken the non-covalent tyrosine–tyrosine interactions and may facilitate release of siRNA at the right time for optimal delivery [22]. However, effective siRNA-mediated gene silencing was observed with large polymolecular aggregates (>500–800 nm) [20]. This feature asks question about suitability of such polyplexes for *in vivo* administration that relies on preparation of concentrated and stable colloids of sizes around 100 nm. We also found tyrosine-modified PEI to have a toxicological profile unsuited for *in vivo* administration.

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In this study, we tuned the chemical structure of self-assembling polymers for *in vivo* administration and focused our efforts to improve the biocompatibility of the siRNA carrier. We synthesized various novel hydrophobic polyethylenimines starting from the commercial branched polymer of 25 kDa. We also evaluated their siRNA delivery efficiency and their toxicological profile and selected N-3-pyridyl, N′-PEI thiourea (πPEI) as the most suitable siRNA vehicle for *in vivo* administration. Single injection of siRNA polyplexes in subcutaneous implanted U87 tumors resulted in a significant siRNA-mediated luciferase silencing at about 30% relative to controls.

2. Materials and methods

2.1. Materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Invitrogen (Cergy Pontoise, France). Branched PEI 25 kDa (40,872-7, batch 09529KD-466) and the other chemicals were purchased from Sigma-Aldrich (St Quentin, France) and were used as supplied. Water was deionized on a Millipore Milli-Q apparatus. Before use, regenerated cellulose dialysis membranes (SpectraPor 4, 12–14 kD, SpectrumLabs) were soaked in MilliQ water (200 mL, 3 times, 8 h each) to remove preservatives. Chemical synthesis and work-ups were performed under a chemical fume hood and plastic tubes were guaranteed RNAse-free by the manufacturers. Experiments involving cell lines were performed according to the biosafety level 2 guidance. NMRI nude mice (strain Crl:NMRI-Foxn1^{nu}) were obtained from Elevage Janvier, Le Genest-St-Isle, France. Animal experimentation was conducted according to French regulations at the Inserm U682 animal facility. HEPES buffered saline ×2 (HBS) contained 20 mM HEPES, 240 mM NaCl and 1.5 mM Na_2HPO_4. Buffered solutions and water were sterilized by filtration through 0.22 μm pore membrane. The polymers and siRNA solutions were prepared using sterile media. All solutions were kept sterile by working under a class II microbiological safety cabinet. UV/Vis analyses were performed on a Shimadzu UV2401PC spectrometer. NMR spectra were performed on a Bruker DPX 400 MHz spectrometer. The modification degree of the polymer was determined relative to ethylenimine (EI) residues by integration of ^{1}H NMR signals and was of 30 +/− 5% for all polymer otherwise indicated.

2.2. Succinimidyl ester of vanillic acid

A solution of N, N′-DiCyclohexylCarbodiimide (DCC) (20 g, 97 mmol) in CH_2Cl_2 (25 mL) was added dropwise at 0–4 °C to a solution of N-hydroxysuccinimide (8.9 g, 77 mmol) and vanillic acid (11.8 g, 70 mmol) in ethylacetate (80 mL)/DMF (20 mL). The reaction mixture was then stirred overnight at room temperature. The DiCyclohexylUrea (DCU) was removed by filtration, washed with ethyl acetate (100 mL) and the combined organic phases were washed with saturated NaCl (100 mL), saturated $NaHCO_3$ (twice 100 mL), saturated NaCl (100 mL) and then dried over $MgSO_4$. The solvent was then removed under reduced pressure to give the product as a yellow solid (16.5 g; 70% yield). ^{1}H NMR ($CDCl_3$) δppm: 2.9 (s, 4H), 3.9 (s, 3H), 6.9 (d, J = 8.4 Hz, 1H), 7.55 (d, J = 2.0 Hz, 1H), 7.75 (dd, J = 2.0 Hz, J = 8.4 Hz, 1H), 8.0 (s, 1H). ^{13}C NMR ($CDCl_3$) δppm: 25.7, 56.2, 112.4, 114.8, 116.5, 125.9, 146.7, 152.2, 161.5, 169.5. ES-MS: (M calculated for $C_{12}H_{11}NO_6$: 265.057) found: 288.049 ($[MNa]^+$).

2.3. Succinimidyl ester of salicylic acid

A solution of DCC (10.9 g, 53 mmol) in CH_2Cl_2 (5 mL) was added dropwise at 0–4 °C to a solution of N-hydroxysuccinimide (6.19 g, 53.8 mmol) and salicylic acid (6.16 g, 44.7 mmol) in DMF (30 mL). The reaction mixture was then stirred overnight at room temperature and the DCU was removed by filtration and washed with ethyl acetate (200 mL). The combined organic phases were washed with saturated $NaHCO_3$ (twice 200 mL), citric acid 5% (200 mL), saturated NaCl (200 mL), dried over $MgSO_4$ and the solvent was removed under reduced pressure to give the product as a yellow solid (10.1 g, 95% yield). ^{1}H NMR ($CDCl_3$) δppm: 2.9 (s, 4H), 6.95 (t, J = 7.2 Hz, 1H), 7.05 (d, J = 8.0 Hz, 1H), 7.58 (td, J = 7.2 Hz, J = 1.6 Hz, 1H), 8.0 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H). ^{13}C NMR ($CDCl_3$) δppm: 25.6, 108.1, 118.0, 120.0, 130.1, 137.9, 161.9, 165.0, 169.1. ES-MS: (M calculated for $C_{11}H_9NO_5$: 235.048) found: 258.038 ($[MNa]^+$).

2.4. Succinimidyl ester of nicotinic acid

A solution of DCC (7.0 g, 33.98 mmol) in CH_2Cl_2 (10 mL) was added dropwise at 0–4 °C to a solution of N-hydroxysuccinimide (4.1 g, 35.6 mmol) and nicotinic acid (3.89 g, 31.6 mmol) in DMF (30 mL). The reaction mixture was then stirred overnight at room temperature and the DCU was removed by filtration and washed with ethyl acetate (200 mL). The combined organic phases were washed with saturated $NaHCO_3$ (twice 200 mL), citric acid 5% (200 mL), saturated NaCl (200 mL), dried over $MgSO_4$ and the solvent was removed under reduced pressure to give the product as a white solid (5.7 g, 80% yield). ^{1}H NMR ($CDCl_3$) δppm: 2.8 (s, 4H), 7.2–7.43 (m, 1H), 8.31–8.35 (m, 1H), 8.82–8.84 (m, 1H), 9.26 (d, J = 1.6 Hz). ^{13}C NMR ($CDCl_3$) δppm: 25.7, 121.7, 123.6, 137.8, 151.4, 155.2, 160.8, 168.9. ES-MS: (M calculated for $C_{10}H_8N_2O_4$: 220.049) found: 221.056 ($[MH]^+$).

2.5. N-3-pyridyl-, N′-PEI–thiourea (πPEI) 1

A solution of 3-pyridyl isothiocyanate (460.4 mg; 3.46 mmol) in CH_2Cl_2 (40 mL) was added dropwise at room temperature to a solution of PEI (500 mg; 11.62 mmol) in CH_2Cl_2 (40 mL). After 30 min, the solvent was evaporated under reduced pressure. The residue was dissolved in water (40 mL) and the solution was adjusted to pH 4.0 by addition of hydrochloric acid 3 M. Dialysis against water (1 L volume; 2 changes over 48 h) and freeze-drying gave 650 mg of the pyridyl PEI–thiourea. The modification degree was estimated at 28% relative to ethylenimine. ^{1}H NMR (D_2O) ∂ppm: 3.95–2.5 (m, 4H, $NHCH_2CH_2NH$), 4.2 (t, 0.5H, CH_2NCS), 7.5 (m, 0.25H, CHaro), 7.86 (m, 0.25H, CHaro), 8.45 (m, 0.5H, CHaro). λ_{max} (ε calculated for ethylenimine unit): 245 nm (2660 $M^{-1}.cm^{-1}$). Average molecular weight (MW): 122.0 g/mol.

2.6. N-3-pyridyl-, N′-PEI–thiourea 15% 2

A solution of 3-pyridyl isothiocyanate (230 mg; 1.73 mmol) in CH_2Cl_2 (50 mL) was added dropwise at room temperature to a solution of branched polyethylenimine (500 mg; 11.62 mmol) in CH_2Cl_2 (40 mL). After 30 min, TLC indicated full consumption of the isothiocyanate. The solvent was then evaporated under reduced pressure. The residue was dissolved in water (40 mL) and the solution was adjusted to pH 4.0 by addition of hydrochloric acid 3 M. Dialysis against water (1 L volume; 2 changes over 48 h) and freeze-drying gave 515 mg of pyridyl PEI-thiourea 15%. ^{1}H NMR (D_2O) ∂ppm: 4.02–2.65 (m, 4H, $NHCH_2CH_2NH$) 4.24 (t, 0.3H, CH_2NCS), 7.82 (m, 0.15 H, CHaro), 9.1–8.1 (m, 0.3H, CHaro). MW: 100 g/mol.

2.7. N-4-aminophenyl, N′-PEI–thiourea 3

A solution of *tert*-butyl 4-isothiocyanatophenylcarbamate (500 mg; 2.02 mmol) in CH_2Cl_2 (50 mL) was added dropwise to a solution of PEI (265 mg; 6.16 mmol) in CH_2Cl_2 (40 mL). The reaction was then stirred for 30 min at room temperature and the solvent was removed by evaporation under reduced pressure. The residue was dissolved in aqueous HCl 3 M (30 mL) and the solution was carefully adjusted to pH 4.0 with aqueous NaOH 6 M. Dialysis against water (2 changes over 24 h) and freeze drying provided N-(4-aminophenyl), N′-PEI–thiourea (650 mg) as a yellow powder. ^{1}H NMR (D_2O) ∂ppm: 4.0–2.67 (m, 3.3H, $NHCH_2CH_2NH$), 4.21 (m, 0.7H, CH_2NCS), 7.35 (bm, 1.4H, CHaro). Average MW: 140 g/mol.

2.8. 4-hydroxybenzamido-polyethylenimine 4

A solution of freshly prepared 4-hydroxybenzoic acid (635 mg; 4.6 mmol) and BOP (1.7 g; 5.06 mmol) in DMF (20 mL) was added dropwise at room temperature to a solution of PEI (1 g, 23.2 mmol) in DMF (50 mL). After 2 h under stirring, DMF was removed by evaporation under reduced pressure. The crude product was taken in water, dissolved by addition of aqueous sodium hydroxide solution 1 M (pH 11) and subjected to dialysis against water (1 L, 2 changes over 24 h). Lyophilization provided 4-hydroxybenzamide-PEI (0.7 g) at a modification degree of 28%. ^{1}H NMR (D_2O) ∂ppm: 2.6 (bm, 2.9H, $-NHCH_2CH_2NH-$), 3.22 (m, 0.55H, Phe-$CONHCH_2CH_2NH-$), 3.35 (m, 0.55H, Phe-$CONHCH_2CH_2NH$) 6.57 (d, $J = 7.3$ Hz, 0.55H, CHaro), 6.97 (m, 0.55H, CHaro).

2.9. Vanillamido-polyethylenimine 5

A solution of succinimidyl ester of vanillic acid (2.8 g, 10.5 mmol) in DMF (20 mL) was added at room temperature to a solution of polyethylenimine (0.9 g, 20.9 mmol in ethylenimine) in methanol (5 mL). After 2 days under stirring, the residue was treated with NaOH 1 M (5 mL) for 1 h at room temperature. The pH of the solution was then adjusted to pH 7.0 by addition of aqueous HCl 0.5 M and the solution was subjected to dialysis against water (1 L, 5 changes over a 48 h period). Lyophilization afforded the product as yellow powder (1.4 g, 67% yield). ^{1}H NMR (D_2O) δppm: 2.0–3.9 (16H), 6.6 (s br, 1H), 6.9–7.4 (m, 2H). ^{13}C NMR (D_2O) δppm: 36.6, 49.9, 56.0, 115.0, 121.0, 146.9, 150.3. λ_{max} (ε calculated for ethylenimine unit): 290 nm (2000 $M^{-1}.cm^{-1}$), 260 nm (4000 $M^{-1}.cm^{-1}$). Average MW: 127 g/mol.

2.10. 2-hydroxybenzamido-polyethylenimine 6

A solution of succinimidyl ester of salicylic acid (1.47 g, 6.2 mmol) in DMF (15 mL) was added at room temperature to a solution of polyethylenimine (0.9 g, 20.9 mmol in ethylenimine) in CH_2Cl_2 (3 mL). Two hours later, the reaction mixture was completed with methanol (20 mL) and stirred for 24 h. The solvents were removed under reduced pressure and the residue was treated with NaOH 1 M (20 mL). The solution was subjected to dialysis against water (1 L, 2 changes over a 24 h period), aqueous HCl (1 L, 6 changes over a 48 h period) and water (1 L, once for 6 h). Lyophilization afforded the product as a powder (1.8 g, 86% yield). ^{1}H NMR (D_2O) δppm: 2.0–3.9 (13.2 H), 6.7–7.6 (m, 4H). λ_{max} (ε calculated for ethylenimine unit): 300 nm (790 $M^{-1}.cm^{-1}$). Average molecular weight: 117 g/mol.

2.11. Nicotinamido-polyethylenimine 7

A solution of succinimidyl ester of nicotinic acid (3.52 g, 16 mmol) in DMF (15 mL) was added at room temperature to a solution of polyethylenimine (1.4 g, 32 mmol in ethylenimine) in CH_2Cl_2 (10 mL). Two hours later, the reaction mixture was completed with methanol (20 mL) and stirred for 24 h. Solvents were removed under reduced pressure and the residue was treated with NaOH 1 M (20 mL). The solution was subjected to dialysis against water (1 L, 2 changes over a 24 h period), aqueous HCl (1 L, 6 changes over a 48 h period) and water (1 L, once for 6 h). Lyophilization afforded the product as a powder (2.2 g, 75% yield). ^{1}H NMR (D_2O) δppm: 2.6–3.9 (10.1 H),7.1–9.0 (m, 4H). λ_{max} (ε calculated for ethylenimine unit): 260 nm (1800 $M^{-1}.cm^{-1}$). Average MW: 112 g/mol.

2.12. Materials for the siRNA delivery experiments

PAGE-purified oligonucleotides terminated with two 2′-deoxythymidines at their 3′-ends were purchased from Eurogentec (Seraing, Belgique), supplied at 100 μM concentration and stored at −20 °C. The egfpluc fusion gene silencing experiments were performed with a RNA duplex of the sense sequence (siluc): 5′-CUU ACG CUG AGU ACU UCG A. Untargeted RNA duplex (sic) was of sequence 5′-CGU ACG CGG AAU ACU UCG A. Polymers were dissolved in water and the solutions were adjusted to pH 6.0 with NaOH 1 M. Solutions were stored at 4 °C. Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere and grown in DMEM medium with 10% Fetal bovine serum (FBS) (Perbio, Brebières, France), 100 units/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine (Eurobio, Courtaboeuf, France). U87 cells (human glioblastoma ATCC HTB-14) were transformed to stably express the *Photinus pyralis* luciferase-enhanced green fluorescence protein fusion gene originating from the pEGFPluc plasmid (Clontech, Mountain View, CA) [23]. The plasmid coded as well for a resistance gene to G418, selection was thus performed by adding to a final concentration of 0.8 μg/mL to the cell culture medium. The day before experiments U87egfpluc cells were seeded into 96-well plates at a density of 5000 cells per well in 100 μL cell culture medium.

2.13. Determination of polyplexes stability

Polyplexes (EI/P of 60) were prepared by mixing the control siRNA (735 ng, 50 pmol) and each polymer (120 nmol in ethylenimine) either in RPMI (15 μL, final pH of 7.8) or in water (15 μL, final pH of 6.0). After 20 min of incubation, the polyplexes were treated with increasing charge excess of heparin, for 30 min, loaded onto a 1.5% 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 μg/mL, 15 min), released siRNA were visualized with a UV transilluminator and quantified using NIH ImageJ analysis software.

2.14. Acido-basic titration of the polymer

The buffering capacities of the polymers were determined using an automatic titrator (Abu 901, radiometer, Copenhagen) and a Mettler Toledo Inlab423 glass electrode. Two mL-aqueous solutions of the polymer (50 mM amino nitrogen atoms) containing 100 mM KCl were titrated with 0.1 M NaOH. The pH-sensible aggregation ability of the polymers was determined by monitoring the turbidity and measuring the pH of a 20 mM aqueous solution of the polymer during an acido basic titration.

2.15. Determination of the polymer toxicity (MTT assay)

MTT assays were performed in triplicate in 96-well plates. Cells (U87efgpluc) were seeded the night before experiment at 8000 cells/well. Different amounts of polymers in a final volume of 11 μL water, were added to the cells to obtain the final concentrations as indicated in the graph. After 48 h incubation, the cell culture medium was removed from each well and replaced with a 0.5 mg/mL MTT solution in DMEM without serum (220 μL). After 2 h incubation at 37 °C, excess reagent was removed by aspiration. The formazan crystals were then dissolved in DMSO (100 μL) and quantified spectrophotometrically in a microplate reader at a wavelength of 570 nm. The cell viability was plotted relative to untreated cells that were grown the same day in the same plate.

2.16. Hemolysis experiments

Before experiments, sheep red blood cells (RBC) (Eurobio, Courtaboeuf, France) were recovered by centrifugation at 400 RCF for 10 min and washed three times with NaCl aqueous solution (150 mM). RBC were then resuspended in phosphate buffer saline (PBS) and plated in 96-well plates at a rate of 15×10^6 cells in 50 μL. Solutions of polymer (50 μL) in PBS at different concentrations were then added to the erythrocytes and incubated for 1 h at 37 °C. The release of hemoglobin was determined after centrifugation at 700 RCF for 10 min by spectrophotometric analysis of the supernatant at 550 nm. Complete hemolysis (100% control value) was achieved using TritonX-100 to a final concentration

of 0.1% w/v. The negative control was defined by suspension of RBC in phosphate buffer alone. The experiments were performed in triplicate.

2.17. Formation of siRNA polyplexes

First procedure (assembly of complex at EI/P of 60 at low concentrations — typical for *in vitro* experiment): a 100 nM solution of siRNA in RPMI medium without serum (40 μL) was added to a 0.5 mL polypropylene tube containing an aqueous solution of the polymer (10 mM in ethylenimine, 0.96 μL). The mixture was then homogenized and used within 2 h. Second procedure (assembly of complex at high concentrations — for *in vivo* experiment): typically for EI/P of 9.4, polyplexes were formed at room temperature by mixing a 100 μM siRNA solution (80 μL) with a 9% (w/v) glucose solution (100 μL). This mixture was then rapidly added to 20 μL of a 150 mM (18.3 mg/mL) πPEI solution, pH 6.0. The same procedure was applied using other medium by replacing the glucose solution with 300 mM NaCl, HEPES buffered saline (HBS) ×2, pH 7.0 and pH 7.8 or lowering the initial siRNA solution to 15.6 μM.

2.18. SiRNA delivery experiments in vitro

For polyplexes prepared according to the first procedure, the complexes (11 μL containing 16.17 ng siRNA, EI/P of 60) were directly added to cells by dilution into each well of the 96-well plate. Polyplexes prepared according to the second procedure were diluted at a 1/50 ratio in 10% FBS cell culture medium. Diluted complexes (10 μL) were then added to the cells. Cells were then let to grow in the incubator without further handling. Luciferase gene expression was usually assessed 48 h after delivery using a commercial kit using manufacturer's protocol (Promega, Charbonnières, France) using 20 μL of lysis buffer. The luminescence was measured from 0.8 μL of lysate during 1 s with a luminometer (Centro LB960 XS; Berthold, Thoiry, France). The error bars represent standard deviation derived from triplicate experiments and luciferase activity was calculated relative to untreated cells.

2.19. Estimation of complex size

After assembly, the complexes (40 μL) were diluted in water (0.5 mL). Dynamic light scattering measurements were performed using a NanoZS apparatus (Malvern instruments, Paris, France) at 25 °C using a refractive index of particles of 1.49. Data were analyzed using the multimodal number distribution software included with the instrument. Diameters of complexes were calculated from the intensity of the signal. Transmission electron microscopy was performed on a Philips CM120 apparatus [24]. EM samples were prepared by transferring polyplexes onto carbon grids (Ted Pella 1822-F) and were stained with uranyl acetate.

2.20. SiRNA delivery experiments in vivo

Particles were freshly prepared according to the second procedure and were injected within 60 min. Male NMRI nude mice (6–8 weeks of age, weights in the 25–30 g) were subcutaneously inoculated in both flanks with 5×10^6 U87egfpluc cells in 100 μL PBS. Mouse behavior and weight were monitored every 2 to 4 days and tumor growth was evaluated with a digital caliper. Tumor volume was calculated using the formula $[(\text{length}\times\text{width}^2)\times0.52]$. Twenty-five days after inoculation, mice were anesthetized using isoflurane and complexes (40 μL) were injected into the tumor mass using a 0.3 mL syringe equipped with a 30 G needle (BD MicroFine, Becton Dickinson, Franklin Lakes, NJ, USA). Average volume of the tumors was 260 mm^3. Mice were anesthetized again 4 days later. The volume of tumors was measured and the animals were sacrificed by cervical dislocation. The tumors were dissected, washed in PBS and cut in two pieces. One piece was fixed in PFA for histological analysis. The other piece was wiped up with an adsorbing paper and freeze-dried in liquid nitrogen. For luciferase activity measurement, the freeze-dried tumor was weighted, diluted in cold PBS at 80 mg of tumor/mL and grinded with a T25 ultra-turrax at 0–4 °C. The solutions were then clarified by centrifugation (10,000 RCF, 10 min) and luciferase activity of the supernatant was measured and expressed as relative light units (RLU) integrated over 10 s and normalized per μg of tumor protein by using the BCA assay (Pierce, Brebières, France). Each condition was performed on group of 5 tumors. Statistical analysis (Student t-test) and calculation of the t probability (p) were performed using KaleidaGraph Software 4.1 on paired data.

3. Results and discussion

3.1. Polymer synthesis and initial in vitro siRNA delivery activities

Most synthetic delivery systems use initial electrostatic anchorage to sulfated (polyanionic) proteoglycans of the external cell surfaces to accumulate in large amounts into endosomes. The use of this route relies on cationic complexes stable enough to sustain electrostatic competition with the external polyanionic cell surface receptors but capable of dissociating inside the cell for payload delivery [25,26]. After cell-anchorage to anionic proteoglycans, polyplexes may be directed into endosomal compartments and sense a pH-decrease from pH 7.4 to 4.5. It has been demonstrated that PEI polyplexes interfere with the normal acidification process [27]. The buffering ability of PEI blocks the cell-induced endosomal acidification at measured pH of 5.9 [21] or 6.1 [27], leading eventually to a rupture of the endosome membrane and subsequent access of the polyplexes to the cytosol. Chemical modification of the water-soluble 25 kDa polyethylenimine (PEI) with tyrosine improves considerably its oligonucleotide delivery abilities at a cellular level [20,28]. Our mechanistic investigation [22] indicated that the efficiency of PEIY may be due to an optimal hydrophobic/hydrophilic balance. We therefore attempted to provide more ground to this assumption as well as to improve the overall siRNA delivery properties of the delivery agent by improving the toxicological profile. At a starting point, we explored variations in the chemical structure of the PEI-grafted hydrophobic domains and in the type of linkage for possible improvement of delivery activity and/or toxicological profile (Fig. 1A).

The commercial branched 25 kDa PEI was chosen as the core cationic element because it readily accepts chemical modification [29]. The various hydrophobic elements were grafted to this cationic core at a ratio of 30% relative to ethylenimine unit (EI) unless indicated. In principle, this value corresponds to a full modification of the PEI primary amines [30]. The first polymer, named πPEI **1** was prepared by reaction with 3-pyridyl-isothiocyanate (0.3 equivalent relative to PEI ethylenimine unit) in a DMF/dichloromethane. The effect of the modification degree was investigated by synthesizing a second polymer, πPEI **2**, with a grafting of 15%. This polymer was prepared using the same protocol but with 0.15 equivalent of isothiocyanate relative to PEI. Polymer **3** was made from N-*tert*-butyloxycarbonyl-4-aminophenylisothiocyanate and obtained after removal of the protecting group with trifluoroacetic acid. Polymer **4** was prepared from 4-hydroxybenzoic acid using benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) as the condensing reagent. Finally, polymers **5**, **6** and **7** were prepared by reacting PEI with succinimidyl esters of vanillic acid, salicylic acid and nicotinic acid, respectively, also in DMF/dichloromethane. After completion of the reactions, each polymer was purified by dialysis and isolated as hydrochloride salts. Yields were typically in the 50–60% range and ^{1}H NMR integration of characteristic peaks confirmed the modification degree to be close to the expected values of 15% for **2** and 30% for all the others. The ability of each polymer to make up electrostatic complexes with oligonucleotides was then assessed by agarose gel electrophoresis. Regardless of the modification, the onset of full complexation happens at EI to oligonucleotide phosphate (P) ratio of 3.0 (Fig. S2, Supporting information).

siRNA duplexes can effectively and selectively silence the expression of a gene only if the duplexes can be transferred into the cell. The delivery abilities of the polymer were hence directly deducted by measuring

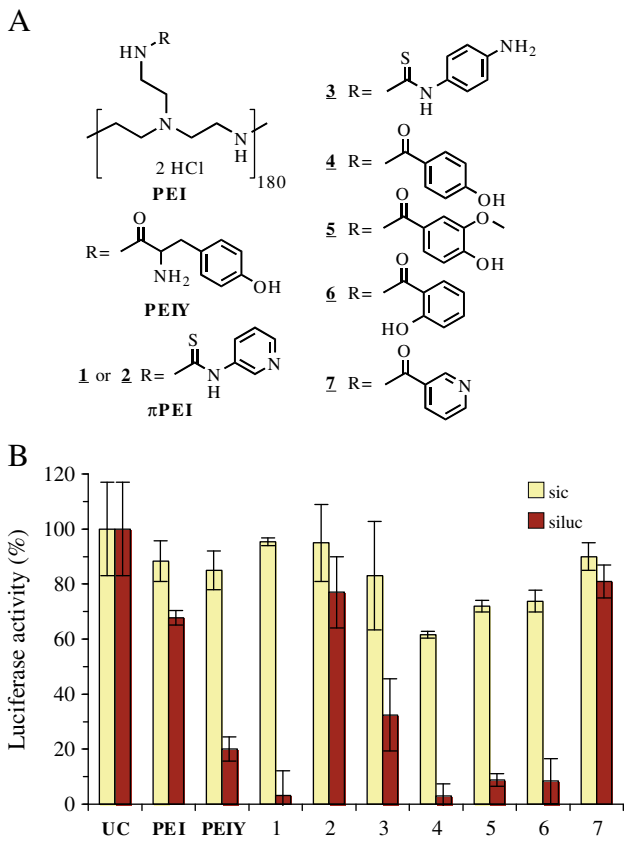


Fig. 1. A. Chemical structure of the siRNA delivery polyethylenimines (PEIs). Polymers were modified at about 30% extend relative to ethylenimine (EI) residues, except for πPEI **2**, which has the same chemical structure of **1** but modified at 15%. B. Efficiency of each polymer to deliver siRNA into U87 cells stably transformed to express an egfpluc fusion protein (U87egfpluc). Each PEI was mixed with either luc-targeting siRNA (siluc) or untargeting siRNA (sic) and added to cells. Luciferase activity was measured 48 h later and plotted relative to untreated cells. Data show the means of triplicate and standard deviations. Final concentrations were 10 nM in siRNA and 24 μM of the indicated polymer to give siRNA polyplexes at a EI/P of 60. UC: untreated cells.

the siRNA-mediated silencing of a luciferase-enhanced green fluorescent chimera gene using either targeting (siluc) or control (sic) siRNAs. To avoid flaws from transient transfection experiment, the human gliobastoma U87 cell line was previously genetically transformed to express a luciferase-green fluorescent protein chimera. The resulting U87egfpluc has the advantage to express a fusion protein that can be silenced with a unique targeted siRNA and be easily quantified either by monitoring the green fluorescent protein or by measuring the enzymatic activity of the firefly luciferase domains. The siRNAs polyplexes were prepared by mixing the polymer with siRNA in RPMI cell culture medium at final concentrations of 240 μM in ethylenimine unit (EI) and 100 nM in siRNA. The ensuing cationic polyplexes with an ethylenimine to siRNA phosphate (EI/P) ratio of 60 were then simply added to cells by dilution to 1/10 with the serum-containing cell culture medium to reach final concentrations of 24 μM EI and 10 nM siRNA. Forty-eight hours later, the luciferase activity of the cell lysate was measured and expressed relative to untreated cells (UC) (Fig. 1B). As previously seen on A549 cells [17], unmodified 25 kDa PEI showed to be a poor *in vitro* siRNA carrier. In contrast, PEIY, πPEI **1** and hydroxybenzamido-PEI derivatives **4**, **5** and **6** enabled a high (above 90%) and selective siluc-mediated luciferase silencing. Decreasing the pyridylthiourea content on PEI (πPEI **2**) considerably diminished the siRNA-mediated luciferase gene silencing while a less dramatic diminution was obtained with the polymer **3**. Replacement of the thiourea linkage of πPEI **1** with an amide bond (polymer **7**) almost abolished the polymer delivery ability. Fig. S1 (Supporting information) showed that the polymers gave a similar siRNA delivery efficiency trend on the A549 cell line.

3.2. Physicochemical properties of the polymers

Cationic delivery systems usually do not promote nucleic acid translocation by direct action on the plasma membrane. They rather divert the cell machinery and enter into endosomal compartments in where they undergo a pH change from 7.8 to as low as 4.5 within minutes. It is generally assumed that the high buffering abilities (the so-called "proton-sponge" properties) of PEI in that range contribute strongly to its nucleic acid translocation efficiency by promoting an osmotic pressure imbalance in between PEI-loaded endosomes and the cytosol, leading eventually to the rupture of endosomal lipid membrane and hence to the desired translocation [21].

We examined whether the observed difference in siRNA delivery properties between the effective polymers (**1**, **3**, **4**, **5**, **6**) and the poorly effective ones (**PEI**, **2** and **7**) was directly due to modified hydrogen buffering properties. In this aim, the pH of 50 mM aqueous solution of the various polymers, was measured upon incremental addition of NaOH (Fig. 2A). The PEI **4** was hardly soluble at concentration above 40 mM and was dropped out from this assay. Polyornithine, a cationic polymer without any nucleic acid delivery ability on its own was used as a control. As expected, the high basicity of polyornithine amines does not enable to buffer the solution pH in the 4.5 to 7.8 range as seen by the sharp increase in the pH solution with as low as 0.04 equivalent of base per monomer (black triangle). Acid base titration of PEI (red square) confirmed the weaker basicity of the ethylenimine residues because as much as 0.2 equivalent of base is required to raise the pH solution from 4.5 to 7.8. Chemical modification of PEI altered the acid base titration profiles but only to a limited extend. In particular, the titration lines of the modified PEIs superposed almost exactly in the pH 6.0–7.8 range and had slopes similar to the PEI one. Considering that PEI blocks endosome near pH 6.0, this experiment strongly indicates that the high siRNA delivery efficiencies of the polymers **1**, **3**, **5** and **6** relative to PEI, **2** or **7** do not directly derived from a peculiar buffering abilities. Nonetheless, each polymer undergoes a variation of its protonation state. From the slope of the acid base titration curve, we can determine that for all polymers, 6% of ethylenimine residues go from neutral to cationic when the pH of the solution decreased from pH 7.8 to pH 6.0. This phenomenon can impact the aqueous solubility of the polymer and was analyzed in the next experiment (Fig. 2B). In here, we determined the pH value at which macroscopic state of the polymer goes from soluble to particulate (Fig. 2B). Aqueous solutions of each polymer were carefully treated with incremental addition of NaOH. The pHs were then measured at the onset of turbidity. The bared PEI never formed a turbid solution, even above pH 9.0 (not shown) and the less effective polymers **2** and **7** precipitated only at pHs above 7.8. Interestingly, the effective siRNA delivery polymers precipitated upon ethylenimine deprotonation within the pH 6.0–7.8 indicating that this pH sensitive aggregation ability is important.

For effective delivery, siRNA polyplexes should remain stable enough to sustain electrostatic competition in extracellular media and conditions. Assembly of the siRNA polyplexes by other means than electrostatic interactions such as hydrophobic ones may actually limit electrostatic-induced dissociation of the complex upon binding with sulfated proteoglycans present on the external face of the plasma membrane. After internalization, siRNA polyplexes should however be able to dissociate for a controlled release of siRNAs. We examined the electrostatic stability of the siRNA polyplexes at pH 7.8 or pH 6.0 to mimic interaction with the sulfated proteoglycans receptors either on the external plasma membrane faces [31] or in PEI-loaded endosomes [21], respectively. The siRNA polyplexes from πPEI **1** and **2**, **6** or **7** were prepared at the same EI/P ratio of 60 which previously used for the *in vitro* delivery assay (Fig. 1). The cationic particles were then incubated with increasing amounts of the sulfated polysaccharide heparin and release of siRNA was quantified from agarose gel electrophoresis assays (Fig. 3).

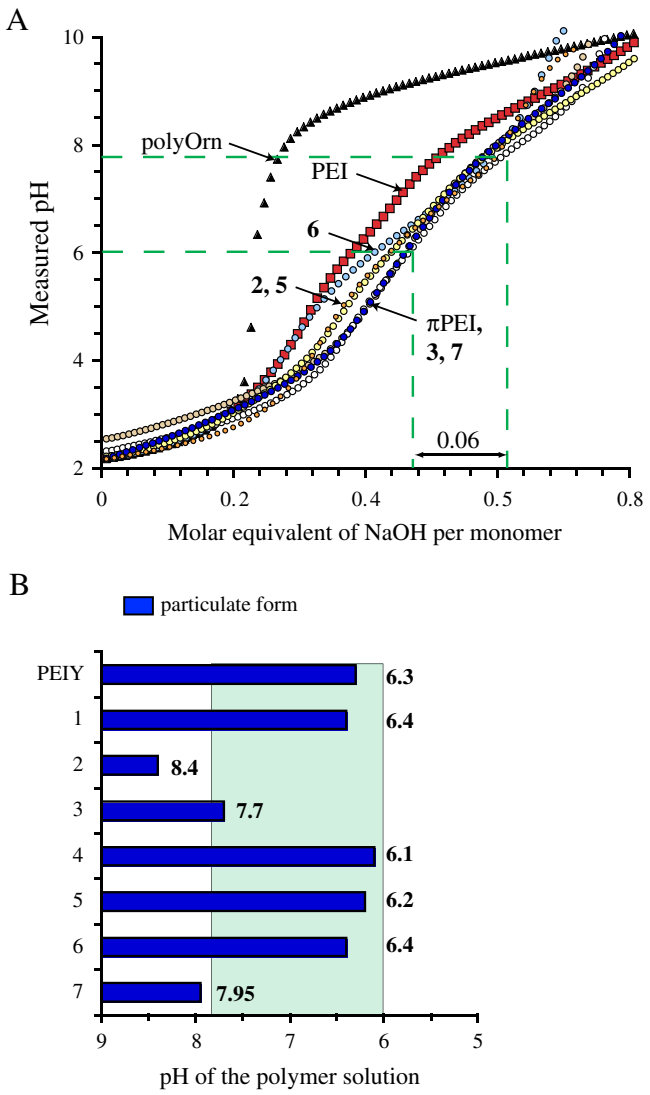


Fig. 2. A. Acido-basic titration of polyornithine and of the various PEI polymers as indicated. B. Sensitivity of the polymers to form particulate matter as a function of the pH. The value indicated the transition pH at which the 20 mM aqueous polymer solution becomes turbid. The colored box indicates the putative cellular pH range encountered by the particles.

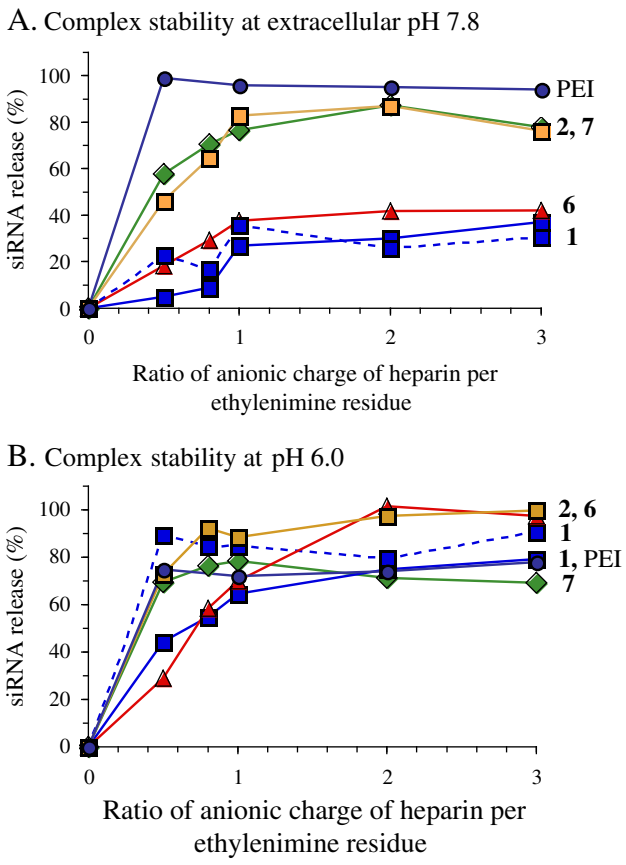


Fig. 3. Electrostatic stability of the polyplexes at pH 7.8 (extracellular value; graph A) and pH 6.0 (PEI-buffered endosomal value, graph B). siRNA polyplexes made from PEI (black dot), πPEI **1** (blue square) and **2** (yellow square), **7** (green diamond) or **6** (red triangle) at EI/P of 60 (full line) or 9.4 (dotted line) were incubated for 20 min with increasing amounts of heparin. Release of siRNA was determined from agarose gel electrophoresis analyses.

The siRNA polyplexes made from the soluble polymer PEI, **2** and **7** dissociated almost quantitatively upon challenge with heparin, at pH 7.8 (Fig. 3A). This suggests that their weak siRNA delivery efficiencies may be due to a too early extracellular siRNA release. In contrast, the polymers πPEI and **6**, which exist in a particulate state above pH 6.4, improved the electrostatic cohesion of the siRNA/πPEI complexes. Decreasing the incubation pH to 6.0 (Fig. 3B), value of measured pH of PEI-buffered endosomes [21] and at which any polymer is or becomes fully soluble (Fig. 2B), led to almost quantitative siRNA release in presence of heparin. Increasing the content of siRNA within πPEI polyplexes by decreasing the EI/P ratio from 60 to 9.4 (dotted line) did not modify the siRNA release profile at both pHs, suggesting that the stability is given by the auto-interaction between the PEI-appended elements.

Altogether, these *in vitro* data were similar to the ones obtained on PEIY [22]. They provided further evidences that the effectiveness of these siRNA delivery polymers is not due to a specific dangling chemical group but is rather related to an overall pH-dependent solubility of the polymer that is tuned to sense the cellular pH variation during the intracellular delivery process.

Cytotoxicity can be a limiting factor for the development of any drug carrier. Polycations and especially the ones used for nucleic acid delivery are known to damage cellular membranes [32,33] and to induce cell death [32]. The potential damaging effect of PEI, **6**, πPEI and PEIY on cellular membranes was estimated from hemolysis assays using sheep red blood cells (Fig. 4A).

The PEI, but also the hydroxyphenyl-grafted **6** and PEIY polymers possess a hemolytic activity at concentrations above 1000 μg/mL. In contrast, the πPEI did not induce any release of hemoglobin from the red blood cells up to a concentration level of 5000 μg/mL. This result suggests that πPEI, which has the same delivery activity as PEIY and **6**, is the most suitable carrier for *in vivo* administration. Next, πPEI cytotoxicity was compared to that of PEIY, **6** and PEI by exposing the U87-egfpluc to increasing concentrations of each polymer. Cell viability was then extrapolated 48 h later by measuring the cellular mitochondrial activity using the MTT reagent (Fig. 4B). Except for **6**, the polymer affected the cell viability with a sigmoidal type dose–response profile, as previously observed on other cell lines [34]. The πPEI polymer appears more innocuous to U87 cells (IC_{50} = 90 μg/mL) than PEIY and PEI (IC_{50} = 40 μg/mL) and onset of toxicity is much above the πPEI final concentration of 3 μg/mL used for siRNA delivery.

Polymer toxicity on cells followed nonetheless the same trend than the hemolytic activity, suggesting that direct PEI-induced damage to membrane is undesired but can be worked out not only by chemical modification of PEI with succinate [14] but also with aromatic groups. The reason behind the singular low hemolytic and cellular toxicity of πPEI is unclear. The most obvious speculation is that the specific

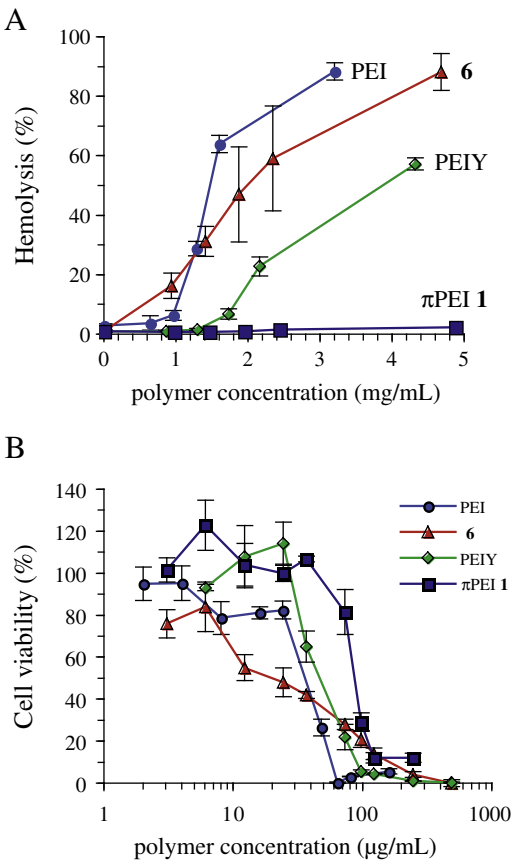


Fig. 4. A. Hemolytic activity of the polymers. 100% hemolysis was obtained using triton X-100 (final concentration of 0.1% w/v) B. Effect of the polymer concentration on the viability of U87egfpluc cells. Cell viability was monitored by measuring the cell metabolic activity using the MTT assay.

polar/hydrophobic nature of thiourea forms a shield around the PEI, hence preventing insertion of ethylenimine residues into lipid bilayers and hence damage.

3.3. Optimization of complexes for in vivo administration

The previous experiments showed πPEI to be an excellent *in vitro* siRNA delivery vehicle with a favorable toxicological profile when formulated in commercial RPMI medium at a concentration of 0.03 mg/mL (0.24 mM in EI). Cumulative experiences with PEI for *in vivo* delivery of nucleic acid suggest to administer nucleic acid polyplexes at a much higher PEI concentration of about 1–2 mg/mL [8,35]. However, the kinetic and polyplex assembly processes depend not only on the incubation medium [36] but also on the concentration of the partners. Concentrated solutions, and especially ones made with self-assembling polymers, can yield to sizes of polyplexes too large for *in vivo* injection. For instance, self-assembly of πPEI in RPMI at 29.3 μg/mL (diluted conditions used for *in vitro* experiments), yielded particles with an average diameter of 240 nm after 30 min incubation. The same polymer, in the same conditions but at higher concentration (1.83 mg/mL) grew faster and ended after 30 min incubation to particles with diameter of 1200 nm. We therefore aimed to find a suitable assembly medium enabling formation of stable πPEI/siRNA polyplexes, even at high concentration. To limit the auto-assembly of the polymer during the siRNA polyplex formation while remaining as close as possible to a physiological pH, the πPEI stock solution (18.3 mg/mL) was adjusted to pH 6.0. The πPEI polymer was then diluted alone or in the presence of siRNA at a final 1.83 mg/mL concentration either in aqueous 4.5% (w/v) glucose solution, in 150 mM NaCl or in HEPES buffered saline (HBS), pH 7.0 or pH 7.8. After a 2-h incubation period at 25 °C, the mixtures were analyzed by dynamic light scattering (DLS) measurements (Fig. 5A). In the electrolyte-free glucose solution, the polymer alone was actually not fully soluble and slightly self-assembled into a population of 21 nm-diameter particles. The presence of siRNA at an EI/P ratio of 9.4 drove the two partners to assemble into larger 96 nm-diameter particles. Increased πPEI to siRNA ratio (EI/P ratio of 60) led to similar particles (138 nm-diameter). Yet, the smaller particles (now 27 nm in diameter), representing presumably the excess of πPEI, were detected again. This type of polyplex formation appears similar to the one described for the soluble PEI [13] but starting with a cationic πPEI polymer being already a nanoaggregate instead of a single molecule and suggests an assembly process essentially driven by electrostatic interaction and a fast kinetic. Presence of salts in the medium and increased pHs led to slightly larger complexes presumably because these elements favor hydrophobic interactions and lower the electrostatic repulsion between cationic particles [35–37]. Before undertaking siRNA delivery experiments, we examined the effect of dilution of the particles in a cell culture medium of higher pH and containing serum. siRNA/πPEI polyplexes of about 100 nm in diameter (prepared in 4.5% glucose, EI/P = 9.4) showed to grow in size over times but at a slow pace. After 4 h, sizes of particles remained below 200 nm (data not shown). Next, the siRNA delivery efficiency of these so formed polyplexes was evaluated *in vitro* on the U87egfpluc cells (Fig. 5B) by dilution into the 10% FBS containing cell culture medium to a final πPEI concentration of 27 μM. Results showed the assembly media, the stoichiometry, and the particle sizes, to have little impact on the final siRNA-mediated luciferase silencing except using HBS pH 7.8, for which luciferase activity diminished to about 20% relative to untreated cells versus less than 10% for the other media.

The concentrated particles prepared in 4.5% glucose appeared the most interesting because of their smallest sizes and highest efficiency. Preparation of particles at EI/P ratio of 9.4 versus 60 appeared also more interesting on a first base because of the occurrence of a single population with the narrower size distribution and slightly highest siRNA-mediated gene silencing. They were hence characterized further. The gel agarose electrophoresis analysis (Fig. 5C) showed the 4.5% glucose solution to permit full electrostatic complexation of the siRNAs by the cationic πPEI above EI/P of 3. Transmission electron microscopy (Fig. 5D) showed 4.5% Glc solution to conduce πPEI and siRNA at an EI/P ratio of 9.4 to assemble into a population of spherical particles with an average diameter of 60.0 nm (SD: 12 nm, n = 40).

The duration of siRNA-mediated luciferase gene silencing after carriage using concentrated πPEI/siRNA polyplexes prepared in 4.5% glc at EI/P of 9.4 was as well evaluated at the cellular level (Fig. 5E). The πPEI showed to assist a selective siRNA-mediated luciferase gene silencing since delivery of an untargeted siRNA (sic) did not diminish the cellular luciferase activity over the experiment time course. Maximum protein activity inhibition (superior to 90%) was reached 48 h after administration and lasted at least 4 days. It is worth mentioning that the luciferase activity in untreated and πPEI/sic-treated wells increased over times at the same rate. This indicates that the polyplexes did not modify the cellular proliferation rate because the amount of luciferase, produced in the U87egfpluc, is proportional to the number of cells.

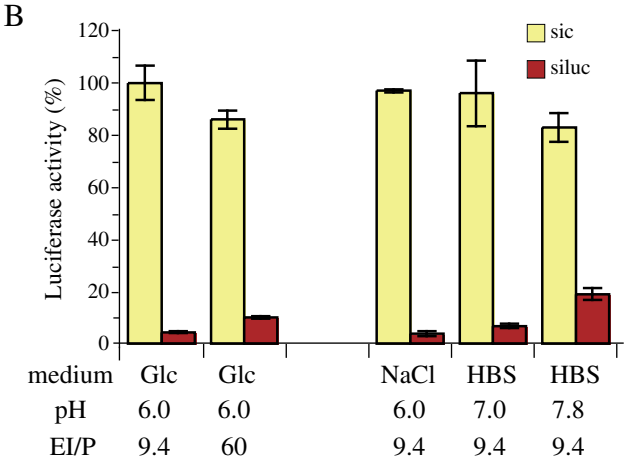
3.4. In vivo experiment

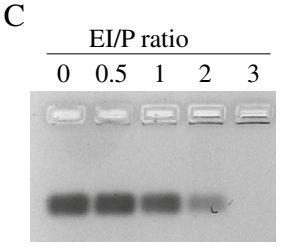
The final experiment was to demonstrate the *in vivo* potential of πPEI to carry any siRNA. Subcutaneous injection of U87egfpluc human glioblastoma cells into flanks of athymic mice led to well-developed solid tumors with exponential growth mode and expressing luciferase. At this development stage, the idea was only to provide evidence of the delivery ability of the polymer in an *in vivo* context. We thought it is reasonable to attempt silencing the innocuous and easy to monitor luciferase reporter gene of U87egfpluc cells instead of silencing a putative gene involved in tumor growth [38].

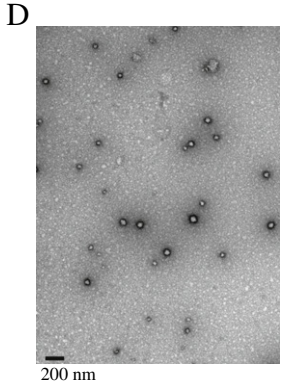
Cationic polyplexes are known to promote erythrocyte aggregation [39], to dissociate in the blood [40], and to accumulate preferentially in lung or liver [41] over tumor, leading to possible systemic toxicity [42]. Before undertaking sophistication by equipping the nucleic acid delivery systems with targeting or/and surface-shielding elements [12, 24], we aimed to prove that the bared cationic self-assembling πPEI polymer conserved its intrinsic siRNA delivery ability into cells also in an *in vivo* setting. As recommended [41], problems related to systemic injection were bypassed by administration of the siRNA polyplexes directly into the tumors. A single dose of siRNA polyplexes was slowly injected into tumors of average volume of 260 mm^3. Four days later, the luciferase activity of the U87egfpluc tumors was measured and reported in Fig. 6. A luciferase gene silencing of 30% was observed with the siluc/πPEI polyplexes by comparison to controls 4 days after injection. A statistical analysis using the Student t-test on paired data indicates a high probability of similarity (p of 0.9) between luciferase expression in the tumor controls and (sic/πPEI)-treated tumors and a significant difference (p of 0.007) between (sic/πPEI)- and (siluc/πPEI)-treated tumors. A 30% gene silencing activity may appear modest but increased dose and design of protocol with repeated injection should help to get better gene silencing activity. Nonetheless this level may be sufficient for cancer immunotherapy where modulation of cytokine level can amplify immune response [38]. If literature led us to believe that chemical sophistication is mandatory for systemic injection, these bared cationic systems may conceivably be already of practical application for intracellular siRNA delivery of accessible tissues such as lung or bladder as previously demonstrated with analogous delivery systems [10, 43].

A

Medium	pH	πPEI/siRNA ratio (EI/P)	Average diameters (nm)	PDI
Glucose (Glc)	6	no siRNA	21	0.35
		9.4	96	0.26
		60	27 (30%); 138 (70%)	0.8
NaCl	6	no siRNA	615	0.35
		9.4	274	0.28
HBS	7.0	no siRNA	147	0.24
		9.4	630	0.8
HBS	7.8	no siRNA	193	0.225
		9.4	700	0.7







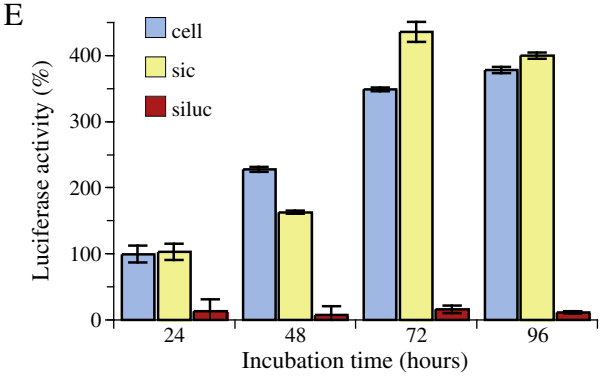


Fig. 5A. Effect of the medium, pH and stoichiometry of the partners on πPEI particles formation. Diameters and polydispersity index were determined from light scattering data. B. Effect of the medium, pH and stoichiometry of the partners on the efficiency of the πPEI polyplexes to deliver siRNA into U87egfpluc cells. Polyplexes were diluted in 10% FBS medium to reach final concentrations of 27 μM πPEI and either 72.7 nM siRNA or 11.4 nM for polyplexes with EI/P of 9.4 and 60, respectively. C. Electrophoresis analysis of the formation of πPEI/siRNA polyplex in 4.5% Glc as a function of the polymer etylenimine monomer (EI) to siRNA phosphate (P) ratio. D. Transmission electron microscopy analysis of πPEI particles at an EI/P ratio of 9.4, prepared in 4.5% glucose. Scale bar represents 200 nm. E. Duration of siRNA-mediated luciferase silencing using πPEI/siRNA polyplexes (EI/P 9.4) prepared in 4.5% glucose (Glc). Luciferase activity was plotted relative to untreated cells of day 1. All particles in this figure were prepared at a πPEI concentration of 1830 μg/mL or 15 mM in EI. NaCl: 150 mM NaCl solution, HBS: HEPES buffered saline.

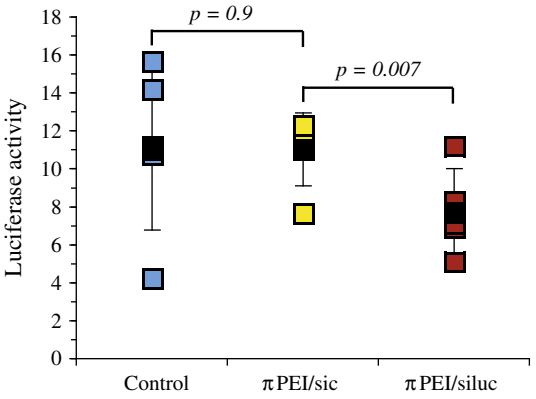


Fig. 6. Luciferase activity of the U87egfpluc tumors 4 days after polyplex injection (in MegaRLU/10 s/μg tumoral protein). Polyplexes (72 μg πPEI, 23.5 μg siRNA in 40 μL 4.5% glucose) were injected into the solid tumor 25 days after subcutaneous inoculation of the U87egfpluc cells. Each condition was performed on group of 5 tumors. Statistical analysis (Student t-test) and calculation of the t probability (p) were performed using KaleidaGraph Software 4.1 on paired data.

4. Conclusion

In summary, we synthesized a novel self-assembling polymeric siRNA carrier by conjugating the branched PEI 25 kDa to pyridines via thiourea linkages. The particular kind of hydrophobicity given by

the pyridylthiourea element appeared to confer to PEI 25 kDa a high siRNA delivery ability *in vitro* and an appreciable low toxicological profile. The πPEI carrier can be formulated with siRNAs to form nanometric delivery systems with sizes and concentrations suitable for local *in vivo* administration. A proof of concept experiment showed already a 30% siRNA-mediated gene silencing 4 days after local intratumoral *in vivo* administration. Our current effort is now directed toward the performance of a comprehensive *in vivo* study.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jconrel.2011.10.007.

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