Self-aggregating 1.8 kDa polyethylenimines with dissolution switch at endosomal acidic pH are delivery carriers for plasmid DNA, mRNA, siRNA and exon-skipping oligonucleotides

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Abstract

Efficiency of polyethylenimine (PEI) for nucleic acid delivery is affected by the size of the carrier and length of the nucleic acids. For instance, PEIs with molecular weights between 10-30 kDa provide optimal DNA delivery activity whereas PEIs with molecular weights below 1.8 kDa are ineffective. The activity of PEI is also severely diminished by substitution of DNA for shorter nucleic acids such as mRNA or siRNA. Here, through chemical modification of the primary amines to aromatic domains we achieved nucleic acid delivery by the 1.8 kDa polyethylenimine (PEI) particles. This modification did not affect the PEI buffering abilities but enhanced its pH-sensitive aggregation, enabling stabilization of the polyplex outside the cell while still allowing nucleic acid release following cellular entry. The aromatic PEIs were then evaluated for their gene, mRNA, siRNA and 2'O-methyl phosphorothioate oligonucleotide *in vitro* transfection abilities. The salicylamide-grafted PEI showed to be a reliable carrier for delivering nucleic acids with cytoplasmic activity such as the mRNA and siRNA or nuclear diffusible oligonucleotide. It was then further equipped with polyethyleneglycol (PEG) and the delivery efficiency of the copolymer was tested *in vivo* for regeneration of dystrophin in the muscle of *mdx* mouse through a 2'O-methyl phosphorothioate-mediated splicing modulation. Intramuscular administration of polyplexes resulted in dystrophin-positive fibers in a mouse model of Duchenne muscular dystrophy without apparent toxicity. These findings indicate that precise modifications of low molecular weight PEI improve its bio-responsiveness and yield delivery vehicles for nucleic acids of various types *in vitro* and *in vivo*.

Keywords

polyethylenimine (PEI); delivery system; transfection; gene delivery; siRNA delivery; mRNA delivery; phosphorothioate RNA; exon-skipping; *mdx* mice; muscular dystrophy

1. Introduction

Nucleic acids are attractive compounds for modulating the cell function with high precision but their poor efficacy in crossing the cell-protective plasma membrane limit their ability to reach the intracellular site of action [1-5]. Modified viruses are on the frontline of DNA delivery for gene therapy [6, 7] but they cannot directly deliver synthetic versions of mRNA, siRNA or oligonucleotides which may exhibit superior activity and minimal immune or toxic response. Some synthetic nucleic acids, administrated alone, were shown to alleviate muscle [8] or liver pathologies [9] but only a small portion of the injected nucleic acids still reaches the cell cytoplasm and/or nucleus. Thus formulation of nucleic acids and their synthetic derivatives with effective carriers remains an interesting approach for gaining efficient and active nucleic acid delivery [8, 10-13]. Among the synthetic carriers, the “proton-sponge” polyethylenimines (PEI) of molecular weights between 10 and 30 kDa have taken a special place because they are reliable *in vitro* transfection reagents which are easily derivatized to achieve desired properties [14-18]. PEI, a cationic polymer, interacts electrostatically with the polyanionic backbone of nucleic acids and enables formation of overall cationic particles entrapping nucleic acids. These cationic polyplexes bind to cell surfaces and are internalized into the endosomal/lysosomal compartment. Active acidification

of this compartment combined with high buffering ability of PEI appears to promote endosomolysis and nucleic acid release into the cytosol. While high molecular weight PEIs have demonstrated *in vivo* nucleic acid delivery activity [19, 20] they suffer from severe limitations. Intravenous administration in mice, at doses that do not promote acute toxicity, leads to rapid accumulation of PEI in the liver with little sign of elimination [21], raising the likelihood of liver poisoning. The undesired liver accumulation of PEI may be partially mitigated by local administration [22] but evidence of damage to lungs [23] or striated muscle [24] was observed as well.

The poor pharmacokinetic profile of polyplexes, largely due to their cationic nature, which nevertheless is necessary for effective cellular uptake, has been addressed by masking the cationic charge with polymers such as the polyethyleneglycol (PEG) [25-27], proteins [28] or hyaluronic acids [29, 30]. Furthermore, enhanced biocompatibility is achieved by novel cationic carriers that have reasonable chance to be eliminated from the animal body either by direct renal clearance or by degradation into inert molecules. Decreasing the molecular weight of PEI from 25 kDa to 2.7 kDa modified the *in vivo* distribution in mice after tail vein injection from liver to kidney accumulation [31]. Also, PEIs with molecular weights less than 2 kDa (referred to here as oligoethylenimines, OEIs) do not exhibit *in vitro* cytotoxicity even at high concentrations [32]. However, OEIs do not possess nucleic acid delivery abilities on their own [33], likely because their sizes (a 1.8 kDa OEI has a degree of polymerization (DP) of 50) do not allow formation of a strong electrostatic association with nucleic acids. OEI or carriers containing ethylenimine units may nonetheless acquire nucleic acid delivery abilities either by modification with hydrophobic domains [34, 35] or by multimerization using disulfide bridges [36, 37].

We have previously enhanced the cohesiveness of siRNA/PEI polyplexes by equipping the 25 kDa PEI with aromatic residues [38, 39] and, by adjusting the hydrophobic/hydrophilic balance of the PEIs, we have succeeded in stabilizing the polyplex in the extracellular space while still allowing siRNA release after cellular entry. Among the modified PEI, a pyridylthiourea-grafted PEI showed high efficiency of siRNA delivery, siRNA-mediated gene silencing in implanted tumors [40], and miR20a-mediated decrease of pro-inflammatory modulators [41]. In a similar fashion, we modified commercial branched 1.8 kDa OEI with various aromatic domains and evaluated their proton buffering, pH-sensitive aggregation and nucleic acid binding abilities. Modified OEI-dependent delivery of nucleic acids of various

lengths and nature (plasmid DNA, mRNA, siRNA and phosphorothioated oligonucleotides) was tested in two types of adherent cell lines (U87 and HeLa). Among the tested compounds salicylamide-OEI was found to be the most efficient in delivering a phosphorothioated RNA in an *in vitro* exon skipping therapy model. This compound was further equipped with methoxyethoxypolyethyleneglycol 550 for polyplex shielding and subsequently evaluated *in vivo* for delivery of an exon skipping-mediating oligonucleotide in the dystrophic *mdx* mouse model upon intramuscular administration.

2. Materials and methods

2.1. General

Water was deionized on a Millipore Milli-Q apparatus. Buffers and water were sterilized by filtration through 0.22 μm pore membrane and were kept sterile by working under a class II microbiological safety cabinet. The branched 1.8 kDa polyethylenimine (referred here as oligoethylenimine or OEI) (reference 408700, batch 0726LH, 50% aqueous solution), methoxyethoxypolyethylene glycol 550 (OEG) and hexane-1,6-diisocyanate were purchased from Sigma-Aldrich (St Quentin, France). Before use, the aqueous solution of OEI was dried by 4 cycles of co-evaporation under reduced pressure with methanol (MeOH) at OEI/MeOH ratio of 1/20 (v/v). All other chemicals were at least of analytical grade and were used as supplied. Chemical syntheses and work-ups were performed under a chemical fume hood. Succinimidyl salicylate and succinimidyl 4-hydroxybenzoate were prepared as previously described [42]. Dialysis membranes were prepared by soaking them in MilliQ water (200 mL, 3 times, 8 h each). Experiments involving cell lines were performed according to the biosafety level 2 guidance. UV/Vis analysis was performed on a Varian Cary 100Bio Spectrometer. NMR spectra were performed on a Bruker DPX 400 MHz spectrometer. The modification degree of the oligomer was determined relative to ethylenimine residues by integration of ^1H -NMR signals and was of 30 $\pm$ 5 % for all oligomers unless otherwise indicated. Quantities of polymers were given in ethylenimine residue. Calculation of the molecular mass (MM) of each OEI was done using the following formula: $\text{MM}_{\text{novelOEI}} = \text{MM}_{\text{OEI}} + C \times \text{MM}_{\text{grafted molecule}}$ with C being the percentage of grafted molecule per ethylenimine. The 5.5 kbp pCMVluc plasmid was amplified by Tebu-Bio (Le Perray-en-Yvelines, France). The 5-methylcytidine, pseudouridine-contained firefly luciferase mRNA (1929nt long, reference L-6107-100, batch T1-AND03A) was purchased from TriLink Biotechnology (San Diego, CA) and was used as

supplied. Control PEI was the 22 kDa jetPEI[®] and was obtained from PolyPlus-Transfection (Illkirch, France). Oligonucleotides were purchased from Eurogentec (Seraing, Belgium) and stored at –20°C as solution in RNase-free water. For oligomer titration by electrophoresis, the oligonucleotide 5'-TCG CCC TGG CCC TCG was labeled on the 5'end with carboxyfluorescein. The siRNAs were annealed at 100 μ M (or 1.5 μ g μ L⁻¹) final siRNA concentration and stored in aliquots at –80°C. Sense sequences of luciferase-targeting and control siRNA duplexes were terminated with two 2'-deoxythymine at the 3'end and were as follows: siLuc: 5'-CUU ACG CUG AGU ACU UCG ATT, siC: 5'-CGU ACG CGG AAU ACU UCG ATT. For the polo-like kinase-targeting siRNA, the sense sequence was: 5'-AGA (U)CA CCC (U)CC U(U)A AA(U) AUU and the antisense was 5'-UAU UUA A(G)G AGG GUG A(U)C UUU. The nucleotide in brackets is a 2'OMe nucleotide [43].

The splice correcting oligonucleotides, active for regeneration of luciferase expression in HeLa705 and the splice switching oligonucleotides active for *mdx* mice, were 2'-O-methyl-phosphorothioate of sequence 5'-CCU CUU ACC UCA GUU ACA (SO-luc) [44] and M23D(+07-18) with the sequence 5'-GGC CAA ACC UCG GCU UAC CUG AAA U-3' (SO-dy) [45], respectively. The SO-dy was used as the control oligonucleotide in experiments using HeLa705.

2.2. Chemical synthesis

2.2.1. Succinimidyl 4-hydroxyphenylacetate

A solution of N,N'-Dicyclohexylcarbodiimide (4.14 g, 20 mmol) in CH₂Cl₂ (5 mL) was added dropwise at 0-4°C to a solution of N-hydroxysuccinimide (2.31 g, 20 mmol) and 4-hydroxyphenylacetic acid (2.55 g, 16.77 mmol) in DMF (20 mL) and ethyl acetate (20 mL). The reaction mixture was then stirred overnight at room temperature. Ethylacetate (150 mL) was added to the mixture and the precipitated dicyclohexylurea was removed by filtration. The organic solution was then washed with saturated aqueous NaHCO₃ (twice 100 mL), citric acid 5% (100 mL), saturated NaCl (200 mL) and dried over MgSO₄. The solvent was finally removed under reduced pressure to give the ester as a yellow solid (4 g, 95% yield). ¹H-NMR (CDCl₃) δ ppm: 2.8 (s, 4H), 3.8 (s, 2H), 6.88 (d, J= 6.4 Hz, 2H), 7.15 (d, J= 6.4 Hz, 2H). ¹³C-NMR (CDCl₃) δ ppm: 25.6, 36.8, 115.7, 122.5, 130.4, 156.2, 167.3, 169.3. ES-MS: (M calculated for C₁₂H₁₁NO₅: 249.0637) found: 272.05392 ([MNa]⁺).

2.2.2. Tyrosine-OEI (YOEI)

A solution of succinimidyl ester of N-Boc-Tyrosine (1.2 g, 3.1 mmol) in DMF (5 mL) was added at room temperature to a solution of OEI (0.33 g, 7.75 mmol in ethylenimine) in MeOH (10 mL). After 2 days of stirring, solvents were removed under reduced pressure and the residue was treated with trifluoroacetic acid (15 mL) for 1 h at room temperature. The excess acid was co-evaporated under reduced pressure twice with ethanol (50 mL). The residue was washed twice with diethylether (50 mL) and taken up in water (10 mL). After full dissolution and addition of aqueous 2M HCl (10 mL), the solution was subjected to dialysis using a SpectraPor 3500 Da membrane against water (1 L, 5 changes over a 48 h period). Lyophilization afforded the product (500 mg, 3.9 mmol in ethylenimine, 50%) as a white powder. A 30% degree of modification relative to ethylenimine residue was determined from NMR integration analysis. $^1\text{H-NMR}$ (D_2O) δppm : 2.7-3.7 (4H, 100%), 4.1 (s broad, 0.3H), 6.8 (s broad, 0.6H), 7.1 (s broad, 0.6H). $^{13}\text{C-NMR}$ (D_2O) δppm : 35.9, 43.5, 47.1, 49.2, 54.7, 116.0, 125.6, 130.9, 155.2, 169.9. λ_{max} (ϵ calculated for ethylenimine unit): 274 ($466 \text{ M}^{-1}.\text{cm}^{-1}$).

2.2.3. N-3-pyridyl-N'-oligoethylenimine-thiourea (πOEI)

A solution of 3-pyridyl-isothiocyanate (365 mg, 2.68 mmol) in CH_2Cl_2 (5 mL) was added at room temperature to a solution of OEI (350 mg, 8.14 mmol in ethylenimine) in CH_2Cl_2 (5 mL). After 1 day of stirring, the solvent was removed under reduced pressure and the residue was taken in 100 mM HCl (20 mL). The solution was subjected to dialysis using a SpectraPor 1000 Da membrane against water (1 L, 2 changes over a 24 h period), aqueous HCl 50 mM (1 L, 6 changes over a 48 h period) and water (1 L, once for 6 h). Lyophilization afforded the product as a yellow powder (0.55 g, 55% yield). A 30.5% degree of modification relative to ethylenimine residue was determined from NMR integration analysis. $^1\text{H-NMR}$ (D_2O) δppm : 2.4-4.3 (4H, 100 %), 7.3 (1H, 7.1%), 7.7 (1H, 7.7%), 8.2 (1H, 7.7%), 8.4 (1H, 7.6%). $^{13}\text{C-NMR}$ (D_2O) δppm : 36.1, 44.5, 48.6, 49.3, 117.2, 120.8, 128.9, 134.9, 171.8. λ_{max} (ϵ calculated for ethylenimine unit): 300 nm ($790 \text{ M}^{-1}.\text{cm}^{-1}$).

2.2.4. 2-hydroxybenzamide-oligoethylenimine (SaOEI)

A solution of succinimidyl-salicylate (1.45 g, 6.1 mmol) in DMF (10 mL) was added at room temperature to a solution of OEI (0.9 g, 20.9 mmol in ethylenimine) in MeOH (40 mL). After

1 day of stirring, the solution was subjected to dialysis using a SpectraPor 1000 Da membrane against water (1 L, 2 changes over a 24 h period), aqueous 50 mM HCl solution (1 L, 6 changes over a 48 h period) and water (1 L, once for 6 h). Lyophilization afforded the product as a yellow powder (1.2 g, 60% yield). A 28% degree of modification relative to ethylenimine residue was determined from NMR integration analysis. $^1\text{H-NMR}$ (D_2O) δppm : 2.4–3.7 (4H, 100%), 6.5–7.7 (m, 4H, 28.5%). λ_{max} (ϵ calculated for ethylenimine unit): 270 nm ($790 \text{ M}^{-1}.\text{cm}^{-1}$). $^{13}\text{C-NMR}$ (D_2O) δppm : 36.1, 44.5, 48.6, 49.3, 117.2, 120.8, 128.9, 134.9, 171.8.

2.2.5. 4-hydroxybenzamide-oligoethylenimine (HBOEI)

A solution of succinimidyl 4-hydroxybenzoate (0.7 g, 2.95 mmol) in DMF (5 mL) was added at room temperature to a solution of OEI (0.45 g, 10.45 mmol in ethylenimine) in MeOH (15 mL). After 1 day of stirring, the solution was subjected to dialysis using a SpectraPor 1000 Da membrane against water (1 L, 2 changes over a 24 h period), aqueous HCl 50 mM (1 L, 6 changes over a 48 h period) and water (1 L, once for 6 h). Lyophilization afforded the product as a yellow powder (0.8 g, 60% yield). A 28% degree of modification relative to ethylenimine residue was determined from NMR integration analysis. $^1\text{H-NMR}$ (D_2O) δppm : 2.2–3.9 (4H, 100%), 6.7 (s, br, 2H, 13%), 7.5 (s, br, 2H, 13%). $^{13}\text{C-NMR}$ (D_2O) δppm : 35.3, 36.3, 43.6, 48.9, 115.4, 129.6, 132.6, 160.6, 165.5. λ_{max} (ϵ calculated for ethylenimine unit): 255 nm ($3500 \text{ M}^{-1}.\text{cm}^{-1}$).

2.2.6. 4-hydroxyphenylacetamide-oligoethylenimine (HPAOEI)

A solution of succinimidyl 4-hydroxyphenylacetate (1.43 g, 5.74 mmol) in DMF (10 mL) was added at room temperature to a solution of OEI (0.817 g, 19 mmol in ethylenimine) in MeOH (40 mL). After 1 day of stirring, the solution was subjected to dialysis using a SpectraPor 1000 Da membrane against water (1 L, 2 changes over a 24 h period), aqueous HCl 50 mM (1 L, 6 changes over a 48 h period) and water (1 L, once for 6 h). Lyophilization afforded the product as a yellow powder (1.2 g, 52% yield). A 30% degree of modification relative to ethylenimine residue was determined from NMR integration analysis. $^1\text{H-NMR}$ (D_2O) δppm : 2.35–3.7 (6 H, 150%), 6.77 (s br, 2H, 16%), 7.06 (s br, 2 H, 16%). $^{13}\text{C-NMR}$ (D_2O) δppm : 36.0, 41.3, 47.8, 49.4, 115.7, 126.4, 130.8, 154.8, 176.7.

2.2.7. Activated OEG

A solution of hexane-1,6-diisocyanate (6.42 mL, 40 mmol) in CH_2Cl_2 (10 mL) was added to methoxyethoxypolyethyleneglycol 550 (OEG) (2.2 g, 4 mmol) in CH_2Cl_2 (20 mL) and the reaction mixture was stirred overnight at room temperature under argon. Hexane (200 mL) was added to the reaction mixture for isolation of the activated PEG as a viscous liquid. The crude product was dissolved again in CH_2Cl_2 (5 mL) and then isolated by addition of hexane (200 mL). This precipitation operation was repeated 3 times. Residues of solvents and hexane-1,6-diisocyanate were then removed under reduced pressure. The isocyanate-activated OEG (1.8g, 2.5 mmol) was obtained as a colorless liquid with a 62% yield. ^1H -NMR (CDCl_3) δ ppm: 1.27 (t, 2H), 1.47 (t, 2H), 3.07 (m, 2H), 3.3 (s, 3H), 3.49 (m, 2H), 3.56 (m broad, 40 H), 4.16 (t, 2H), 4.89 (s broad, 1H). ^{13}C -NMR (D_2O) δ ppm: 26.3, 29.8, 40.8, 58.9, 63.7, 69.6, 70.5, 71.9, 156.4. ES-MS: (M calculated for $\text{C}_{29}\text{H}_{56}\text{N}_2\text{O}_{13}$: 640.35) found: 615.0 ($[\text{MH}_3\text{-CO}]^+$), 659.0 ($[\text{M}(\text{C}_2\text{H}_4\text{O})_1\text{H}_3\text{-CO}]^+$), 703.2 (max intensity $[\text{M}(\text{C}_2\text{H}_4\text{O})_2\text{H}_3\text{-CO}]^+$), 747.2 ($[\text{M}(\text{C}_2\text{H}_4\text{O})_3\text{H}_3\text{-CO}]^+$), 791.2 ($[\text{M}(\text{C}_2\text{H}_4\text{O})_4\text{H}_3\text{-CO}]^+$), 637.0 ($[\text{MH}_2\text{Na-CO}]^+$), 681.0 (max intensity, $[\text{M}(\text{C}_2\text{H}_4\text{O})_1\text{H}_2\text{Na-CO}]^+$), 725.0 ($[\text{M}(\text{C}_2\text{H}_4\text{O})_2\text{H}_2\text{Na-CO}]^+$), 769.2 ($[\text{M}(\text{C}_2\text{H}_4\text{O})_3\text{H}_2\text{Na-CO}]^+$).

2.2.8. SaOEIOEG

A solution of succinimidyl-salicylate (344 mg, 1.45 mmol) in DMF (2.25 mL) was added to a solution of OEI (190 mg, 4.32 mmol ethylenimine) in methanol (3 mL) and the mixture was stirred overnight. The isocyanate-activated OEG (196 mg, 292 μmol) in CH_2Cl_2 (2 mL) was added at room temperature to the reaction mixture. After 6 hours of stirring, the solvents were removed under reduced pressure and the residue was dissolved in 0.1 M aqueous HCl (50 mL). The solution was subjected to dialysis using a SpectraPor 1000 Da membrane against water (1 L, 2 changes over a 24 h period), aqueous 50 mM HCl solution (1 L, 6 changes over a 48 h period) and water (1 L, once for 6 h). Lyophilization afforded the product as a white solid (1.2 g, 60% yield). The degrees of modification relative to ethylenimine residue were determined at 28 and 5% for salicylamide and OEG, respectively from NMR integration analysis. ^1H -NMR (D_2O) δ ppm: 2.4-4.0 (PEI, 14.3H), 3.6 (EG, 8.8H), 6.5-7.7 (m, 4H,). λ_{max} (ϵ calculated for ethylenimine unit): 270 nm ($790 \text{ M}^{-1}.\text{cm}^{-1}$).

2.3. Acido-basic titration of the oligomer and determination of the pH aggregation

The buffering capacities of the oligomers were determined using an automatic titrator (Abu 901, radiometer, Copenhagen) and a Mettler Toledo Inlab423 glass electrode as described [46]. Two milliliters aqueous solutions of the oligomer (50 mM amino nitrogen atoms, hydrochloride salt) containing 100 mM KCl were titrated by incremental addition of 0.1 M NaOH (0.01 mL). The onset of pH-induced aggregation was determined by monitoring the turbidity of 20 mM aqueous solution (0.8 mL) during an acido-basic titration by incremental addition of 1 M NaOH.

2.4. Characterization of polyplexes

2.4.1. Electrophoretic analysis

Fluorescent oligonucleotides (0.5 µg, 1.5 nmol in phosphate) in 50 mM HEPES-Na buffer, pH 7.4 were incubated for 15 min with increasing amounts of each OEI (0, 0.75, 1.5, 3, 4.5, 6, 7.5, 9 nmol in ethylenimine residue) in a final volume of 20 µL. The mixtures were then subjected to electrophoresis in a 1% agarose gel for 20 min at 90 V using 40 mM Tris acetate, 1 mM EDTA solution, pH 8.0 as running buffer. The fluorescent 15 nt-oligonucleotides inside gels were visualized using a BioRad GelDocXR+ imager.

2.4.2. Particle size measurements

Sizes of aggregates/polyplexes were evaluated by dynamic light scattering (DLS) measurements using a NanoZS apparatus (Malvern instruments, Paris, France) at 25°C and a refractive index of particles of 1.49. Data were analyzed using the multimodal number distribution software included with the instrument. Sizes were measured 15 min after assembly and solutions were prepared as follows: for the oligomer/DNA and oligomer/mRNA complexes (N/P 20), each oligomer (60 nmol in ethylenimine residue), buffered to pH 6.0, was mixed with the pCMVluc plasmid (0.99 µg, 3 nmol in phosphate) or luciferase mRNA (1.05 µg, 3 nmol in phosphate) in 150 mM NaCl solution (0.2 mL). Samples were then transferred to a disposable micro-cuvette (reference 759210, Brand GmbH, Wertheim, Germany) for analysis. For the other conditions, each OEI (100 nmol in ethylenimine) was diluted in RPMI cell culture medium (0.2 mL, no serum present) without or with siLuc siRNA (23.8 pmol, N/P 100) or 2'-O-Methyl-phosphorothioate oligonucleotide SO-luc (555 pmol, N/P 10). Sample for transmission electron microscopy (TEM) was prepared as described [47]. Images were taken using a Philips CM-120 apparatus.

2.5. Cell culture

HeLa (ATCC CCL-2), U87 (human glioblastoma ATCC HTB-14), HeLa705 and U87egfpluc cell lines were maintained at 37°C in a 5% CO₂ humidified atmosphere and were grown in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) (Perbio, Brebières, France), 100 units/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine. U87Luc cells (human lung carcinoma; CCL-185; ATCC) were transformed to stably express the photinus pyralis luciferase and a resistance gene against G418. HeLa705 are HeLa cells that were stably transfected with a luciferase gene in which the coding region is interrupted by a mutated β -globin intron [44]. Experiments involving cell lines were performed under biosafety level-2 conditions. For delivery experiments, cells were seeded the day before either in 24-well plates (Costar) (60 000 cells in 0.9 mL 10% FBS-containing cell culture medium) or in 96-well plates (Greiner, BioOne) (5000 to 8 000 cells/well in 0.09 mL 10% FBS-containing cell culture medium). For determining luciferase activity, the cell culture medium was removed and the adherent cells were lysed using a commercial buffer (Promega, Cergy-Pontoise, France) (20 µL per well for 96-well plates, 100 µL per well for 24-well plate). Aliquots were then withdrawn for luciferase activity measurement using a commercial kit (Promega, Cergy-Pontoise, France) and a Centro LB960XS luminometer (Berthold, Thoiry, France). The protein content of cell lysates was determined as well from aliquots using the bicinchoninic acid assay (BCA Uptima, Interchim, Montluçon, France). The results were expressed as relative light units (RLU) integrated over 1 s per nanogram of cell protein lysate (RLU/s/ng of protein). The errors bars represent standard deviation and were calculated from triplicate experiments.

2.6. Determination of the polymer toxicity (MTT assay)

MTT assays were performed in triplicate in 96-well plates. HeLa cells were seeded the day before experiment at 6000 cells/well (0.1 mL). Different amounts of polymers in a final volume of 10 µL water were added to the cells by dilution with the cell culture medium at the final concentrations indicated in the graph. After 24 h incubation, the cell culture medium was replaced with a 0.5 mg/mL MTT solution in DMEM without serum (120 µL). After 2 h incubation at 37°C, the solutions were carefully removed by aspiration. DMSO (100 µL) was added to the cell monolayers to dissolve the formazan crystals and absorbance of solutions was quantified in a microplate reader at a 570 nm wavelength. The cell viability was plotted

as percentage relative to untreated cells that were grown the same day in the same plate. A similar protocol was used for determining the complex toxicity by substituting the polymer for PS-SO or PS-SO containing complex. Complexes were prepared as described in 2.10.

2.7. DNA delivery experiments

Cells were seeded in triplicate in 24-well plates. Aqueous solutions of the various OEIs (9 or 18 μ l of 20 mM aqueous solution for N/P 10 and 20 respectively or 180 or 360 nmol in ethylenimine) were added into 1.5 ml polyethylene tubes. Each polyplex was then formed by addition of the pCMVluc plasmid DNA in 150 mM NaCl (300 μ L containing 6 μ g DNA or 18 nmol in phosphate) After gentle agitation and incubation for 15 min, DNA polyplexes (100 μ L/well) were added to cells by dilution with 10% FBS-containing cell culture medium. Cells were then put back in the incubator and gene delivery was evaluated 24 h later by measuring the luciferase gene activity after cell lysis.

2.8. mRNA delivery experiments

The procedure is described for cells that were seeded in 96-well plates with 90 μ L 10%FBS-containing cell culture medium. The luciferase 5-methylcytidine, pseudouridine-contained mRNA (1.8 nmol in Phosphate, 0.6 μ g in 30 μ L of 150 mM NaCl solution) was added to each derivative (18 or 36 nmol in ethylenimine, 0.9 or 1.8 μ l of 20 mM aqueous solution for N/P 10 and 20 respectively) in a 0.5 ml polyethylene tube. After gentle agitation and incubation for 15 min, each mRNA polyplex (10 μ L) was added to cells by dilution with 10% FBS-containing cell culture medium. Cells were then put back in the incubator and luciferase activity was evaluated 24 h later after cell lysis.

2.9. siRNA delivery experiments

U87egfpluc were seeded the day before transfection in 96-well plates (8 000 cells in 90 μ L 10% FBS-containing cell culture medium). A solution of siRNA in RPMI medium without serum (4 pmol in 20 μ L) was added into a 0.5 mL polypropylene tube containing each oligomer (20 nmol, 1 μ L of a 20 mM solution in ethylenimine). After gentle agitation and incubation for 15 min, the siRNA polyplexes (5 μ L) were added to the cells by dilution with the cell culture medium containing serum. The cells were incubated for 48 h without further change. The luciferase activities were then measured and data was plotted relative to untreated cells.

HeLa cells were seeded the day before in 12-well plates onto cover glasses (50 000 cells in 0.9 mL 10%FBS-containing cell culture medium). A 200 nM solution of siRNA in RPMI without serum (16 pmol in 80 μ L) was mixed with SaOEI (80 nmol, 4 μ L of 20 mM solution) and added to the cells by dilution with the cell culture medium. The cells were incubated for 48 h without further change. The nuclei were then stained with Hoechst 33242, the medium was carefully removed and the adherent cells were washed once with PBS (1 mL) and fixed with 4% paraformaldehyde in PBS (1 mL) for 1 h. The cells were then visualized using a fluorescent microscope.

2.10. Exon skipping experiments

HeLa705 cells were seeded in 96-well plates (Costar) (8 000 cells in 0.1 mL 10% FBS-containing cell culture) 24 h prior to transfection. The SO-luc (or SO-mdx) in RPMI medium without serum (111 pmol in 20 μ L, 5.5 μ M concentration) was added into 0.5 mL polypropylene tube containing each oligomer (20 or 40 nmol in ethylenimine from 20 mM solution). After a 15 min incubation time, the complexes (5 μ L) were added to the cells by dilution with the cell culture serum containing medium and the cells were incubated for 24 h. The luciferase activity was then measured and data was plotted relative to untreated cells.

2.11. *In vivo* experiments

Care and manipulation of mice were performed in accordance with national and European legislations on animal experimentation. The experiments were approved by Essonne veterinary office, France. Mice were anesthetized by intraperitoneal injection of 100 mg of ketamine and 1 mg of xylazine per kilogram of body weight. The dystrophin deficient *mdx* mouse, which carries a nonsense mutation in exon 23 of the dystrophin gene, was used as model for DMD. Experiments were done with 9 to 14 week old *mdx* mice. The SO-dy oligonucleotide (5 μ g) was mixed with SaOEI at N/P 10 or SaOEI-OEG at N/P 5 in 15 μ L 5% glucose. The complexes and the oligonucleotide alone (5 μ g in 5% glucose) were then injected at day 0, 7 and 15 in the tibialis anterior muscle (TA). The contralateral TA muscles were used as control. Mice were sacrificed by cervical dislocation at day 21. The left and right tibialis anterior muscles were collected, frozen in liquid nitrogen-cooled isopentane and preserved at -80°C for histological analysis of 8 μ m transverse frozen sections. Immunodetection of dystrophin was with primary anti-dystrophin NCL-Dys 2 antibody (1:20, Novacastra) followed by secondary antibody conjugated to peroxidase. The dystrophin was revealed in brown using DAB as substrate for the peroxidase.

3. Results

3.1. Chemical modification of OEIs

The commercially available branched 1.8 kDa oligoethylenimine (OEI) was equipped with aromatic elements that render the 25 kDa PEI insoluble at pH > 6.0 [42] (Figure 1). The 3-pyridyl-isothiocyanate was reacted with the primary amines of OEI to yield pyridylthiourea-grafted OEI (π OEI). The tyrosine-grafted OEI (YOEI) was prepared by coupling *N*-*tert*-butoxycarbonyl-protected tyrosine to OEI followed by removal of the protecting group with trifluoroacetic acid. Finally, succinimidyl esters of salicylic acid, 4-hydroxybenzoic acid, and 4-hydroxyphenylacetic acid were reacted with OEI and led respectively to salicylamide-OEI (SaOEI), 4-hydroxybenzamide-OEI (HBOEI) and 4-hydroxyphenylacetamide-OEI (HPAOEI). Each molecule was purified by dialysis in aqueous media and lyophilized as hydrochloride salts with yields in the 50-60% range. ^1H -NMR integration of characteristic signals showed modification degree to be close to 30%, a value corresponding to a complete modification of the OEI primary amines [48].

Figure 1. Chemical structure of OEIs.

3.2. Physicochemical properties of OEIs and polyplexes

The buffering ability of PEI in the pH 6.0-7.6 range was shown to heavily contribute to its transfection efficiency. This function is triggered after polyplex endocytosis which perturbs the ATPase-mediated vacuolar acidification of PEI-loaded endosomes [49]. It was suggested

that PEI and the ATPase vacuolar proton pump are directly responsible for the vacuolar membrane disruption because they build an osmotic pressure within the endosome that might rupture the vacuolar lipid membrane. Given the importance of the PEI proton sponge property, we performed an acido-basic titration for each oligomer and plotted the data as presented in Figure 2A. The results show that at pH <6 the acid-base titration curves for oligomers vary but that at pH >6 these differences are attenuated and the curves have similar slopes and almost superpose. Since the intracellular pH is between 6.0 and 7.6 it follows that oligomers in this group have similar buffering abilities in the biologically relevant intracellular pH range. For each oligomer about 25 to 30% of the ethylenimine residues are protonated (or cationic) at pH 7.6. At pH 6.0, the percentage of cationic amine increased to 40-45%.

A. Acido-Basic titration

B. pH-dependent aqueous solubility

C.

	OEI	YOEI	π OEI	SaOEI	HBOEI	HPAOEI
Aggregation pH	-	>7.0	>5.6	>6.0	>6.4	>6.6

Figure 2. **A.** Acido-basic titration of the OEIs. Initial OEI concentrations were 50 mM in ethylenimine. **B.** Evaluation of the pH value that promotes aggregation in water. Drawing shows an OEI grafted with hydrophobic domains (blue box) and explains how pH variation modifies solubility. Experiments show aqueous solution of OEI to remain clear regardless the pH. Solution of SaOEI turned from clear to turbid between pH 6 and 6.4. **C.** Data reporting the pH at which turbidity/aggregation started for each OEI.

We examined pH-dependent solubility of the oligomers in water by carefully titrating acidic solutions of each oligomer with NaOH, which was stopped when the solution became turbid (Figure 2B). Unmodified OEI remained fully soluble regardless of its protonation state even

at pH 8.5. SaOEI started to aggregate above pH 6.0 and reached maximal turbidity at pH 6.4. Figure 2C shows the initial aggregation of the remaining oligomers from this group at 7.0 for YOEI, 6.6 for HPAOEI, 6.4 for HBOEI, 6 for SaOEI and 5.6 for π OEI. Further increase of pH by 0.4 led to maximal turbidity for all studied oligomers. To sum up, all oligomers were fully insoluble above pH 7.4 and became fully soluble below pH 5.6. This is the pH range encountered during trafficking of the complexes from the outside cellular space (pH 7.4) to PEI-buffered endosomes (pH 5.9) [49].

Figure 3. A. Electrophoretic analysis of polyplex formation at various ethylenimine Nitrogen (N) to nucleic acid Phosphate (P) ratios. **B.** Size evaluation by Dynamic Light Scattering (DLS) of each polymer alone (white bar) or after mixing with plasmid DNA (black bar, N/P 20 in 150 mM NaCl) mRNA (blue dots containing bar, N/P 20 in 150 mM NaCl), siRNA (red triangle containing bar, N/P 100 in cell culture medium) and 2'-O-Methyl-Phosphorothioated Single strand Oligonucleotide (PS-SO, green diagonal hatched bar, N/P 10 in cell culture medium). OEI alone, OEI/PS-SO and OEI/siRNA did not form particles with measurable sizes. **C.** Electron microscopy image of uranyl acetate-stained [SaOEI/PS-SO] polyplexes (N/P 10, prepared in 5% glucose).

To evaluate formation of different OEIs-nucleic acid polyplexes, fluorescently labeled single-stranded oligodeoxynucleotides (SO) were mixed with OEIs at increasing OEI Nitrogen (N)

to nucleic acid phosphate (P) N/P ratios in HEPES-buffered solution, pH 7.4 and the complex formation was analyzed by agarose gel electrophoresis. The water soluble OEI and insoluble YOEI at pH 7.4 at a similar N/P ratio of 3 supported full electrostatic complexation of the oligonucleotide presumably due to OEI's primary amines. Likewise, insoluble π OEI, SaOEI, HBOEI and HPAOEI formed complexes but at N/P ratios ≥ 3 , likely because 30% of the cationizable ethylenimine were transformed to bear non-ionic hydrophobic domains. Small leakage of oligonucleotide is observed from HBOEI and SaOEI formed complexes even at N/P ratio of 6. This phenomenon may be due to oligonucleotide not being fully entrapped within the polymer aggregate and remaining bound at the surface weakly enough to be separated during the electrophoresis analysis.

Before delivery experiments, the assembly media were selected to minimize the effect of the nucleic acid type on the final particle's sizes and subsequently a potential contribution of the particle's size during the transfection experiments. The different OEIs were mixed with various intracellular functional nucleic acids: a plasmid DNA, a mRNA, a siRNA or a 2'-O-Methyl-Phosphorothioated Single-stranded Oligonucleotide (PS-SO). The long plasmid DNA and mRNA were assembled with each polymer in 150 mM NaCl; the siRNA and the PS-SO were assembled in cell culture medium. Sizes were determined from dynamic light scattering (DLS) data and were compared to polymer self-aggregates (Figure 3B). OEI alone, or mixed with either the 21 nt long siRNA duplex or the 18 nt PS-SO, did not form assemblies large enough for detection of scattered lights and were not plotted in Figure 3A. Larger assemblies with apparent radii of 140-160 nm were observed only with the longer and highly negative 1900 nt mRNA and 5500 pb plasmid DNA. For the hydrophobic, uncomplexed OEIs, apparent sizes were seen in the 180-240 nm range. The sizes of the particles were not dramatically modified by electrostatic interactions with nucleic acids, regardless of nature and length of the latter, suggesting that hydrophobic interactions drive particle formation. Finally, transmission electron microscopy (TEM) was performed on [SaOEI/PS-SO] polyplex (N/P 10) following uranyl acetate staining (Figure 3C). The complexes appear to be heterogeneous, displaying an intermediate spherical to fibrous morphology.

3.3. *In vitro* delivery activities

3.3.1. Cytotoxicity

HeLa cells were treated with OEIs diluted in serum-containing cell culture medium and their viability was assessed by MTT assay at 24 h. (Figure 4). As previously reported, the 1.8 kDa OEI was essentially non-toxic, even at concentration as high as 2 mM (or 86 $\mu\text{g/mL}$). The IC_{50} of YOEI, SaOEI and HBOEI was 600 μM (46 $\mu\text{g/mL}$), that of πOEI 800 μM or 64 $\mu\text{g/mL}$, and HPAOEI was the least toxic in this group, with an IC_{50} of 1100 μM or 90 $\mu\text{g/mL}$.

Figure 4. Effect of carrier concentration on the viability of HeLa cells after 24 h incubation in 10% serum containing cell culture medium. Cell viability was quantified by measuring the cell metabolic activity using the MTT assay. Data represent means and standard deviation of triplicate.

3.3.2. Plasmid DNA and mRNA transfection

Intracellular delivery into HeLa and U87 cells of 5500 pb plasmid DNA carrying firefly luciferase under the control of the strong cytomegalovirus promoter was evaluated using OEI-DNA polyplexes assembled in 150 mM NaCl at N/P ratios of 10 and 20. Polyplexes diluted with serum-containing cell culture medium were added directly to cells and luciferase activity was measured 24 h thereafter. The white bars in Figure 5 show the transfection activity of each OEI relative to jetPEI[®] (a linear PEI with a MW around 20 kDa) a reagent known for its high DNA transfection efficiency. In both cell lines the unmodified OEI was not an efficient plasmid DNA carrier as seen previously [33]. The πOEI , SaOEI and HBOEI were weakly

active, YOEI and HPAOEI were moderately active. Only HPAOEI displayed delivery activity that was comparable to that of commercial PEI but relies on polyplexes with increased N/P ratios (10 and 20 respectively for transfection of HeLa and U87 cells, versus 5 for the 22 kDa PEI). OEI-driven delivery of a synthetic chemically stabilized 1900 nt long luciferase mRNA was tested under conditions described above except that Lipofectamine 2000 was used as control because PEI is less effective [50, 51]. The black bars in Figure 5 show that SaOEI was the only polymer with appreciable mRNA delivery activity. The measured activity was equivalent or three times lower than Lipofectamine for the HeLa and U87 cells, respectively.

Figure 5. Plasmid DNA and mRNA delivery activities in **A.** HeLa and **B.** U87 cells. UT: untreated cells, TR: commercial transfection reagents: jetPEI® for plasmid transfection, Lipofectamine® for mRNA transfection. Complexes were prepared by mixing each carrier with the plasmid pCMVLuc or a chemically stabilized luciferase mRNA at N/P ratio of 10 or

20 and added directly to the cells by dilution in the cell culture media containing 10% serum. Final concentrations were 6 μ M in nt, 60 or 120 μ M OEI derivative (in ethylenimine) for N/P 10 or 20. Luciferase activity from the plasmid (white bar) or the mRNA (black bar) was measured 1 day after addition of the complex. For each experiment, complexes were directly diluted in cell culture media containing 10% serum. Data represent means and standard deviation of triplicate.

3.3.3. siRNA transfection

Figure 6. siRNA delivery activity. **A.** siRNA-mediated luciferase silencing in U87 cell line that was stably transformed to express the luciferase reporter gene. UT: untreated cells, siLuc: luciferase-targeting siRNA, siC: control siRNA, siPLK: polo-like kinase-targeting siRNA. Final concentrations were 50 μ M OEI derivative, 12 nM siRNA. Luciferase activity

was measured 2 days after addition of the complex. Activity was reported relative to untreated cells. Data represent means and standard deviation of triplicate. **B.** siRNA-mediated PLK1 silencing in HeLa cells. Images were taken 2 days after addition of a complex at final 80 μ M SaOEI and 20 nM siRNA. Before imaging, cells were fixed with 4% paraformaldehyde and the nuclei were stained with Hoechst 33242. For each experiment, complexes were directly diluted in cell culture media containing 10% serum.

To test OEI driven siRNA delivery (Figure 6) the polymers were mixed with a control siRNA or a luciferase-targeting siRNA and the complexes were added to U87 cells that stably expressed luciferase (U87egfpluc) at a siRNA concentration of 12 nM. After 48 h incubation, the luciferase activities were quantified and data were reported relative to untreated cells (Figure 6A). The results show siRNA-mediated luciferase silencing as follows: π OEI 95%, HBOEI 88%, SaOEI 86%, HPAOEI 50%, YOEI 18%, and OEI 0%. The siRNA-delivery activity of SaOEI was verified in HeLa cells by silencing expression of the Polo-like kinase 1 (PLK1) using a PLK1-targeting siRNA (siPLK). This endogenous protein has a crucial role during cell mitosis and the siRNA-mediated PLK1 silencing was shown to block fast dividing cells in prophase [43]. The cells were incubated untreated, in presence of naked siPLK siRNA or SaOEI/siRNA complexes containing either a control siRNA (siC) or the siPLK. After 2 days of incubation, the nuclei were stained with Hoechst 33242 and the cells were imaged using a fluorescent microscope for analysis of the nuclei (Figure 6B). Naked siPLK and SaOEI/siC did not alter the cell and nucleus morphology relative to untreated cells whereas SaOEI/siPLK complexes promoted the characteristic blockage of >95% of the cells in prophase.

3.3.4. Phosphorothioated oligonucleotide transfection for exon skipping

The process of pre-mRNA splicing into active mRNA can be modulated using oligonucleotides and this approach has been applied to redirect alternative splicing [52] and to induce mRNA repair in, for example rare genetic diseases such as β -thalassemia or Duchenne muscular dystrophy (DMD) [53]. We examined the capacity of OEIs to deliver phosphorothioated oligoribonucleotides that are able to modulate the maturation of pre-mRNA in the HeLa luc705 model of thalassemic pre-mRNA splicing defect [44]. This cell line stably expresses a luciferase pre-mRNA consisting of 2 luciferase exons and a human beta-globin intron. This intron has the peculiarity to carry a point mutation which interferes with correct pre-mRNA splicing, leading to cell without luciferase. Blocking of this mutation

with an oligonucleotide (SO-Luc) restores correct splicing, producing luciferase mRNA and protein (Figure 7A). HeLa luc705 cells were treated with OEI-oligonucleotide (correcting or control) polyplexes and the luciferase activity was assayed at 24 h post-treatment. Tyrosine-grafted 25 kDa PEI (PEIY), which has demonstrated efficiency for delivering the SO-Luc in the same cell line [54] was used as an external control. Among the oligomers, SaOEI was the most effective, a feature that was already observed for the mRNA and siRNA delivery experiments (Figure 7B). Although SaOEI was 30% less efficient than PEIY in restoring luciferase activity it is worth noting that, in contrast to PEIY, SaOEI did not trigger unspecific luciferase activity. The effect of each complex on the cell viability at concentrations and conditions used for the phosphorothioated oligonucleotide delivery experiment was also evaluated using the MTT assay. The result, represented in the Figure 7B by the black dots, showed that most complexes did not impact the cell viability. A 30% decrease in cell viability was only observed with π OEI/SO-Luc complex at a N/P of 20.

A. Scheme of the HeLa Luc705 model for assaying splice correction

B. Efficiency results

Figure 7. Activity of the carriers to deliver a phosphorothioated splice-correcting oligonucleotide (PS-SO) in HeLa Luc705 cells. **A.** Schematic view of HeLa Luc705 splice correcting model. The cell line stably expresses a pre-mRNA that mature into a defective luc mRNA. Selective binding of a splice correcting oligonucleotide onto the pre-mRNA reshuffles the pre-mRNA splicing toward a corrected luciferase mRNA and generates cell luciferase activity. **B.** Efficiency of the various polymers for assisting oligonucleotide-mediated splice correction (left axis, white and red bars for SO-Control and SO-Luc) and estimation of the cell viability using the MTT assay (right axis, black dots). Final concentrations: 260 nM oligonucleotide, 50 and 100 μ M OEI derivative for N/P 10 and 20, respectively.

3.4. *In vivo* exon skipping experiments

Duchenne muscular dystrophy is a X-linked, lethal disorder caused by mutations in the dystrophin gene. Lack of a functional dystrophin results in progressive muscle weakness, cardiomyopathy and early death. Oligonucleotide-induced splicing manipulation has been shown to restore expression of partially functional dystrophin and ameliorate the course of the disease in mouse and dog models. This type of manipulation has been evaluated in recent clinical trials with varying results. While phosphorothioate based compound targeted to exon 51 in DMD affected patients was found to be ineffective [55] a morpholino based oligonucleotide targeted to the same exon performed better and in fact that drug, named Eteplirsen or Exondys 51 was recently approved by FDA [56]. Considering the efficiency of SaOEI *in vitro* for PS-SO delivery, we tested this polymer for splicing manipulation in the *mdx* mouse, an animal model of DMD using a 2'-O-Methyl phosphorothioate (SO-dy) described for inducing skipping of exon 23 and restoring muscle functions of the treated mice [45]. An initial experiment was conducted with polyplexes between the oligonucleotide SO-dy (5 μ g) and SaOEI, which were prepared in 5% glucose solution at N/P 10. Three injections were performed at day 0, 7 and 14 into the tibialis anterior muscle of *mdx* mice and the muscle fibers were analyzed histologically 21 days after delivery to detect the fibers that were immunoreactive to an anti-dystrophin antibody (supporting information, Figure S1). Results indicated that SaOEI/SO-dy treatment did not induce more dystrophin-positive fibers than untreated muscles (average of 30 with n=2 for SaOEI versus an average of 28 with n=4 for untreated muscles). The ability of SaOEI to deliver oligonucleotide into muscle was disappointing but it was comparable to results described using the high molecular weight PEI [57]. It was interesting to note that this treatment did not lead to an important infiltration of mononuclear cells (Supporting information, Figure S1). This is in sharp contrast to

observations made following intramuscular administration of high molecular weight PEIs [58, 59].

Previous studies showed that appending low molecular weight PEG or amphiphilic pluronic to PEI enhances the efficiency of non-viral vector for carrying phosphorothioated oligonucleotide in mouse muscle [57, 60]. The SaOEI (25% salicylamide per ethylenimine) was equipped with methoxyethoxypolyethyleneglycol 550 (OEG; average degree of polymerization of 11) at 5% relative to ethylenimine residue using hexane-1,6-diisocyanate as a linker (Figure 8A). At this level of OEG modification, the SaOEIOEG maintained its nucleic acid binding ability with a full complexation at N/P ratio of 5 and above 5 (Supporting information, Figure S2) but did not lead to nucleic acid transfection in HeLa cells (Data not shown). The SO-dy alone (5 μ g), or the SaOEIOEG/SO-dy complex in 5% glucose solution (N/P 5) with measured average diameter of 170 nm, were administrated again into the tibialis anterior muscle of *mdx* mice (thrice 5 μ g SO-dy at days 0, 7 and 15). Twenty-one days after injection, analyses showed again no sign of infiltration by mononuclear cells in both experiment (Figure 8, images B and C). We then counted the dystrophin-positive fibers, which are detected from negative fibers by an all around and continuous membrane staining. Naked oligonucleotide (Figure 8B) led to an average of 164 dystrophin positive fibers (n=2, 168 and 160 dystrophin-positive fibers per muscle), above published data of 50 to 75 dystrophin-positive fibers in similarly treated *mdx* mice [57, 60]. The SaOEIOEG complex (Figure 8C) led to about 260 dystrophin-positive muscle fibers (n=2; 230 and 296 of dystrophin-positive fibers per muscle), corresponding to a correction of 13% of the total fiber number.

A. Chemical structure of SaOEIOEG

B. (SO-dystrophin)-treated muscle

C. (SaOEIOEG/SO-dystrophin)-treated muscle

Figure 8: *In vivo* efficiency of SaOEIOEG to deliver the intron 23 splice-switching phosphorothioated oligonucleotide (SO-dy) in *mdx*-fiber. **A.** Chemical structure of SaOEIOEG. **B.** (SO-dy)-treated muscle of *mdx* mice. **C.** [SO-dy/SaOEIOEG]-treated muscle. The oligonucleotide alone (5 μg SO-dy) or in complex with SaOEIOEG at a N/P of 5 in 5% glucose were injected at day 0, 7 and 15 into the tibialis anterior muscle of *mdx* mice (n=2). The muscle fibers were analyzed for dystrophin by immunodetection 21 days after the first injection. Fibers are dystrophin-positive when a continuous heavy dark line is seen all around

the fiber. Positive fibers were marked (*) in the magnified pictures.

4. Discussion

Improving the ability of nucleic acid delivery to properly answer to the encountered cellular barriers and environmental variations is important for facilitating nucleic acid therapy. We have previously equipped the 25 kDa branched PEI with aromatic domains to enhance the cohesiveness of siRNA polyplex in extracellular spaces/media while still allowing siRNA release after cellular entry [42]. In this study, we modified 1.8 kDa PEI with the same aromatic domains we used previously on the 25 kDa. Each carrier had a similar buffering ability in the encountered physiological pH range and maintained a proton-sponge ability presumed to favor endosomolysis [40]. The water solubility of the different hydrophobic OEIs was assayed at various pHs. The results show that the hydrophobic OEIs were fully insoluble above pH 7.4 and became fully soluble at pH below 5.6. These pH switches occur at pHs encountered inside endosomal compartments and suggest that these hydrophobic OEIs may enhance cohesiveness of complexes outside the cells but not inside endosomes. According to our hypothesis, they should therefore possess nucleic acid delivery activity. Examination of their nucleic acid delivery activities showed indeed that these hydrophobic OEIs with dissolution switch at endosomal pHs were nucleic acid carriers. Interestingly to note, the transfection efficiency did not entirely depend on the carrier. The nature of the nucleic acid to be delivered appears to contribute as well. The HPAOEI (aggregation pH>6.6) was the most effective plasmid DNA carrier but was a much less efficient carrier for mRNA, siRNA or PS-SO. The salicylamide-OEI (SaOEI, aggregation pH>6.0) did not deliver plasmid DNA but was able to carry all the 3 other studied nucleic acids with good to excellent efficiency. The π OEI (aggregation pH>5.6) was seen to be specific for siRNA delivery and was actually the most efficient for this purpose. YOEI (aggregation pH>7.0) and HBOEI (aggregation pH >6.4) were seen as active carriers but lag behind the others in each transfection experiment. Observation of a marked difference in efficiency with different nucleic acids is not so surprising because the transfection activity relies on polyplex formation, which is based on electrostatic nucleic acid/carrier and hydrophobic carrier/carrier interactive binding modes. Varying the length of the nucleic acids, and hence number of negative charges from 11000 to 18, do not dramatically impact the assembly process as seen by the low difference in the size of particle. However, the hydrophobic/electrostatic balance

within the polyplex is different and likely impact the polyplex trafficking, disassembly and payload release within the cell [61].

Among the various tested carriers, the salicylamide-modified OEI showed to be a reliable carrier for delivering nucleic acids with cytosolic activity (mRNA, siRNA) or oligonucleotide capable of diffusing freely inside the cell nucleus (the exon-skipping-promoting PS-SO) [62]. It was then further examined for its ability to assist regeneration of dystrophin-positive fiber in the muscle of *mdx* mice following intramuscular injection. Even if the SaOEI was a poor carrier for oligonucleotide delivery in muscle, its presence appears to be tolerated as its intramuscular administration did not promote mononuclear infiltration in contrast to PEI-based polyplex [24]. Its shielding with PEG afforded an effective non-viral vector for delivering oligonucleotides into muscle's fiber. The number of generated dystrophin-positive fibers (about 260) were 2-fold below the published number using PEI-PEG copolymer (570 dystrophin-positive fibers) [60] and similar to the most efficient pluronic-PEI copolymer (about 250 dystrophin-positive fibers) [57]. These results are promising because the chemical structure of the copolymer SaOEIOEG was not finely optimized.

4. Conclusion

In conclusion, our results prove that adequate modification of 1.8 kDa OEI with different aromatic domains is an efficient approach to convert the starting inefficient soluble polymer into a nucleic acid carrier. Physicochemical analysis showed that the hydrophobic OEIs with dissolution switch at endosomal pHs are good to excellent carriers for nucleic acids with cytosolic activities. Shielding of the salicylamide-OEI with polyethyleneglycol render the cationic carrier efficient for delivering 2'-O-methyl phosphorothioated oligonucleotide in muscle via intramuscular administration. Studies can now be designed to finely adjust the chemical structure and physicochemical properties of the carriers for improving the delivery efficiency and the tolerability of the delivery system toward intravenous administration.

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Appendix A. Supplementary data

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Supporting information

Self-aggregating 1.8 kDa polyethylenimines with dissolution switch at endosomal acidic pH are delivery carriers for plasmid DNA, mRNA, siRNA and exon-skipping oligonucleotides

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Content :

The supporting data section contains 2 figures

In vivo activity: intra-muscle administration of SO-dy or SaOEI/SO-dy in dystrophic (mdx) mice. Immunohistology analysis of muscle fibers at +21 days

Figure S1. *In vivo* activity: Efficiency of SaOEI to deliver the intron 23 Splice-Switching oligonucleotide (SO-dy) in *mdx* muscle fibers. Experiments were conducted with the oligonucleotide in complexed form with SaOEI at N/P 10. The delivery was performed by three injections done at day 0, 7 and 15 into the tibialis anterior muscle of *mdx* mice (n=2). The muscle fibers were analyzed histologically 21 days after delivery. The presence of dystrophin is revealed by the presence of brown line around the muscle fibers (*).

Figure S2. Agarose gel electrophoresis of polyplex showing formation of electrostatic polyplexes between SaOEIOEG and a fluorescently-labeled oligonucleotide (5'-FAM-TCG CCC TGG CCCTCG). The fluorescent oligonucleotides ($0.5 \mu\text{g}$, 1.5 nmol in phosphate) in 50 mM HEPES-Na buffer, pH 7.4 were incubated for 15 min with increasing amounts of SaOEIOEG. The polyplexes were then analyzed by electrophoresis in a 1.5% agarose gel and the fluorescence was detected using a UV transilluminator.