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for

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

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Materials. Succinimidyl ester of N-Boc protected α-amino acids were purchased from Novabiochem, Merck KgaA (Darmstadt, Germany). Branched PEI (40,872-7, batch 09529KD-466; 25 kDa) was from Aldrich (St Quentin, France). Water was de-ionized on a Millipore Milli-Q apparatus. All other chemicals were at least of analytical grade and were used as supplied. PAGE-purified siRNA duplexes (20 µM solution in DEPC-treated water) were purchased from Eurogentec (Seraing, Belgium) and stored at -20°C. Silencing RNA duplexes were terminated at 3'-ends by overhang of two 2'-deoxythymidines. The luciferase gene originating from pGL3 plasmid was silenced with a RNA duplex of the sense sequence : 5'-CUUACGCUGAGUACUUCGA. Untargeted RNA duplex was of sequence 5'-C**G**UACGC**G**GA**A**UACUUCGA (bold marks showed mismatches by comparison to pGL3 *luc* sequence) and endogenous lamin A/C gene was silenced with a RNA duplex of sense sequence 5'-CUGGACUUCCAGAAGAAC.[1] Cell culture media were from Eurobio (Courtaboeuf, France) and foetal bovine serum (FBS) was from Perbio (Brebieres, France). G418, and Lysis and luciferin solutions for monitoring luciferase activity, were purchased form Promega (Charbonnières, France).

UV/Vis analysis was performed on a Varian Cary 100Bio Spectrometer. NMR spectra were performed on Bruker DPX 300 or 200 MHz spectrometers. Interferin (Polyplus-transfection, Illkirch, France) was used according to manufacturer's protocol. Before use, dialysis membranes were soaked in Milli Q water (200 mL, 3 times, 8 h each) to remove preservatives. Transmission electron microscopy analyses were performed on a Philips CM-12 apparatus. The luminescence was measured using a luminometer (Centro LB960 XS; Berthold, Thoiry, France). The buffering capacities of the polymers were determined using an automatic titrator (Abu 901, radiometer, Copenhagen), a Mettler Toledo Inlab423 glass electrode. 2 mL-aqueous solutions of the polymer (50 mM amino nitrogen atoms) containing 100 mM KCl were titrated with 0.1 M NaOH. PEI and PEIL remained soluble over the full experiment whereas insoluble materials were observed for PEIY and PEIF above pH 6.5, and PEIW above pH 5.0.

Synthesis

Note: The amino-acid content of the polymer was determined relative to ethylenimine residues by integration of ^{1}H NMR signals. Quantities of polymers were also given in ethylenimine residue. Calculation of the molecular mass of the novel polymers was done using the following formula:

$MM_{PEIaa} = MM_{PEI} + C/100(MM_{aa} - MM_{water})$

C: percentage of amino-acid content per ethylenimine.
MM: molecular mass

Boc protected-PEIY. A solution of succinimidyl ester of *N*-Boc-tyrosine (2.35 g, 6.2 mmol) in DMF (5 mL) and CH_2Cl_2 (5 mL) was added at room temperature to a solution of polyethylenimine (0.53 g, 12.3 mmol in ethylenimine) in CH_2Cl_2 (3 mL). After overnight stirring, the polymer was precipitated by addition of ethyl acetate (250 mL). The residue was dissolved in 0.1 M aqueous HCl (150 mL), subjected to dialysis using a SpectraPor 12-14 kDa membrane against water (1 L, 5 changes over a 48 h period) and lyophilized to obtain the Boc-TyrPEI, with a tyrosine content of 30% per ethylenimine (1.15 g, 76%) as a white powder. ^{1}H NMR (CD_3OD) δ 1.38 (s, 9H), 2.2-3.0 (12H), 4.2 (br s, 1H), 6.75 (br s, 2H), 7.05 (br s, 2H).

PEIY-HCl. N-Boc-Tyr-PEI (1.05 g, 7.5 mmol in ethylenimine) was treated with trifluoroacetic acid (5 mL) for 1h at room temperature. The excess acid was rotary evaporated. The residue was washed twice with diethylether (50 mL) and taken up in water (10 mL). After full dissolution, the solution was completed with aqueous 2 M HCl (10

mL) and 5 M NaCl (2 mL) and the milky solution subjected to dialysis using a Spectra-Por 12-14 kDa membrane against water (1 L, 5 changes over a 48 h period). Lyophilization afforded a residue that was dissolved in 0.2 M HCl (50 mL). The solution was lyophilized again to afford the product (835 mg, 6.5 mmol in ethylenimine, 86 %) as a slightly yellow powder. ^{1}H NMR (D_2O) δ 2.2-3.7 (13H), 4.2 (br s, 1H), 6.8 (br s, 2H), 7.1 (br s, 2H). ^{13}C NMR (D_2O) δ 36.05, 36.2, 43.9, 47.6, 49.6, 55.0, 116.3, 125.8, 131.25, 155.6, 170.2. λ_{max} (ε calculated for ethylenime unit): 274 (466 $M^{-1}.cm^{-1}$).

PEIF-HCl. A solution of succinimidyl ester of *N*-Boc-phenylalanine (1.2 g, 3.3 mmol) in DMF (3 mL) was added at room temperature to a solution of polyethylenimine (0.3 g, 6.9 mmol in ethylenimine) in CH_2Cl_2 (1 mL). After 2 days' stirring, solvents were removed under reduced pressure and the residue was treated with trifluoracetic acid (2 mL) for 0.5 h at room temperature. The excess acid was coevaporated under reduced pressure twice with ethanol (50 mL). The residue was washed twice with diethyl ether (50 mL) and taken up in water (10 mL). After full dissolution, the solution was completed with aqueous 2 M HCl (10 mL) and 5 M NaCl (2 mL) and the milky solution subjected to dialysis using a SpectraPor 12-14 kDa membrane against water (1 L, 5 changes over a 48 h period). Lyophilization afforded a residue that was dissolved in 0.2 M HCl (30 mL). The solution was lyophilized again to afford the product (305 mg, 2.2 mmol in ethylenimine, 31 %) as a white powder. ^{1}H NMR (D_2O) δ 2.4-3.7 (13H), 4.2 (br s, 1H), 7.1-7.5 (m, 5H). ^{13}C NMR (D_2O) δ 36.09, 37.09, 43.9, 47.6, 49.4, 55.0, 128.5, 129.9, 130.30, 134.2, 170.25. λ_{max}(ε calculated for ethylenime unit): 210 (5400 $M^{-1}.cm^{-1}$).

PEIW-HCl. The succinimidyl ester of *N*-Boc-Trp (1.8 g, 3.3 mmol) was added at once to a solution of polyethylenimine (0.28 g, 6.6 mmol in ethylenimine) in DMF (18 mL). After overnight stirring, the solution was diluted with 10 mL 2 M HCl and subjected to dialysis using a SpectraPor 12-14 kDa membrane against water (1 L, 5 changes over a 48 h period). Lyophylisation was followed by a treatment with trifluoracetic acid (10 mL) for 4 h at room temperature. The excess acid was rotary coevaporated twice with ethanol (50 mL). The residue was washed twice with diethylether (50 mL), taken up in 2 M HCl (20 mL) and subjected to dialysis using a SpectraPor 12-14 kDa membrane against water (1 L, 3 changes over a 48 h period). Lyophilization of the soluble fraction afforded the product (0.6 g, 4 mmol in ethylenimine, 60 %) as a white powder. ^{1}H NMR (D_2O) δ 1.12 (1H), 2.0-3.9 (m, 14H), 4.1 (br s, 1H), 6.5-7.6 (m, 5H). ^{13}C

NMR (D_2O) δ 27.2, 29.4, 44.0, 47.7, 54.3, 106.5, 112.6, 118.7, 120.17, 122.5, 125.6, 127.3, 136.6, 170.5. λ_{max}(ε calculated for ethylenime unit): 280 (1800 $M^{-1}.cm^{-1}$).

PEIL-HCl. The succinimidyl ester of *N*-Boc-leucine (1.0 g, 3.0 mmol) was added at once to a solution of polyethylenimine (0.26 g, 6 mmol in ethylenimine) in DMF (10 mL). After overnight stirring, the solution was diluted with 10 mL 2 M HCl and lyophilized. The residue was treated with trifluoracetic acid (2 mL) for 4 h at room temperature. The excess acid was then coevaporated under reduced pressure twice with ethanol (50 mL). The residue was washed twice with diethylether (50 mL), taken up in 2 M HCl (20 mL) and subjected to dialysis using a SpectraPor 12-14 kDa membrane against water (1 L, 3 changes over a 48 h period). Lyophilization afforded the product (267 mg, 1.9 mmol, 31 %) as a white powder. ^{1}H NMR (D_2O) δ 0.85-0.95 (m, 6H), 1.6-1.9 (m, 3H), 2.7-3.8 (m, 10H), 4.1 (br s, 1H). ^{13}C NMR (D_2O) δ 21.5, 22.36, 24.35, 36.4, 40.27, 44.01, 47.9, 49.8, 52.4, 171.75.

Size of particles. The apparent sizes were determined via dynamic light scattering measurements using a Malvern nanoZS apparatus with the following specifications: sampling time = 90 s; refractive index of medium = 1.3402; refractive index of particles = 1.47; medium viscosity = 1.145 cP temperature = 25°C. Data were analyzed using the multimodal number distribution software included with the instrument. ζ potentials were measured with the same apparatus and with the following specifications: 20 measurements per sample; dielectric constant = 79; temperature = 25°C; beam mode FZKa) = 1.5 (Smoluchowski model). Complexes (1 mL volume) were prepared as described for delivery experiment.

Electron microscopy analysis. Samples were transferred onto ultrathin carbon film grids (Ted Pella, 1822-F, formvar removed) by placing the grid on top of 10 µL drop for 1 min. Grid with adherent particles was wicked from one side, placed on 100-µL water drop for 30 s for washing, wicked, placed for 1 min on 60-µL drop of freshly filtered 1.33% uranyle acetate, wicked again and air dried before viewed.

Agarose gel electrophoresis analysis. 15 µL RPMI solutions containing GL2luc siRNA (3.3 µM) and increasing amounts of cationic vectors were subjected to electrophoresis in a 1.2% agarose gel containing 1 mM EDTA and 40 mM Tris acetate buffer pH 8.0. RNA was visualized with a UV transilluminator after staining in a ethidium bromide solution (0.5 µg/mL).

Cell culture. A549 cells (human lung carcinoma; CCL-185; ATCC) were transformed to stably express the Photinus pyralis luciferase gene originating from the pGL3 plasmid (Clontech, Mountain View, CA) and a resistance gene against G418 according to the described procedure.[2] The only difference was that cells were originally transfected with JetPEI (PolyPlus-transfection, Illkirch, France). Cells were grown in RPMI medium supplemented with 10% FBS and antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin and 0.8 µg/mL G418). HeLa cells (human cervix epithelial adenocarcinoma; CCL-2; ATCC) were grown in Eagle's MEM medium supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 µg/mL). Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere and all experiments were done in triplicate. The day before experiment, cells were seeded in 24-well plates at 25000 cells/well in fresh complete medium (0.5 mL).

Complexes formation for siRNA mediated gene silencing. The procedure is for 24-well plates experiment. Typically, an aqueous solution of the polymer-HCl salt (10 mM in ethylenimine; 12 nmol, 1.2 µL) was added to RPMI medium (100 µL) containing 6 pmol (88 ng) of siRNA. After agitation, the complexes were incubated for 30 min at room temperature and added in each well by dilution with the cell medium containing serum (0.5 mL). 24 h later, and to ensure optimal cell growth, each well was completed with an optional addition of cell medium containing serum (0.5 mL). The gene expression profiles were analyzed 48h after addition of the complexes. The amount of polymer or lipid corresponds to an optimal determined empirically.

Quantification of the luciferase gene production. Luciferase gene expression was determined 48h after delivery with a commercial kit using manufacturer's protocol (Promega, Charbonnières, France). The luminescence was measured from 1 µL of lysate during 1s with a luminometer (Centro LB960 XS; Berthold, Thoiry, France). Luciferase activity was expressed as light units integrated over 10 s (RLU) and normalized per mg of cell protein by using the BCA assay (Pierce, Brebières, France). The errors bars represent standard deviation derived from triplicate experiments. Luciferase-silencing efficiency of GL3luc was calculated relative to untreated cells. We also systematically checked that untargeted siRNA was not effective in gene silencing.

Determination of the lamin A/C gene production. Lamin A/C gene silencing was monitored 48 h after transfection by immunofluorescence. Mouse monoclonal lamin

A/C specific antibody (clone X-67; RDI, Flanders, USA) was used at 1/4 dilution and rabbit polyclonal NuMA antibody (GeneTex, San Antonio, Texas, USA) was used at 1:500 dilution. Cells grown in four-well-chambered cover glasses (Lab-Tek; Nunc, Roskilde, Denmark) were fixed for 10 min on ice with methanol cooled at -20°C. Target gene specific and control primary antibodies were added and incubated for 1 h at 4°C. After washing in PBS-BSA 1%, Cy3-conjugated anti-rabbit (GeneTex) and FITC-conjugated anti-mouse antibodies (Calbiochem, Darmstadt) were added and incubated for 1h at 4°C. Cells were finally stained for 5 min at room temperature with Hoechst 33342 (1 µM in PBS) and embedded in Moewiol.

Determination of the polymer toxicity (MTT assay). The MTT assay was performed in triplicate. A549luc cells were seeded into 96-well plates at a density of 5000 cells per well (100 µL cell culture medium). 24 h later, the polymers (20 µL), at different concentrations, were added to obtain a final concentration of 10, 20, 40 and 80 µM. 24 h later, and to ensure optimal cell growth, each well was completed with an optional addition of cell medium containing serum (100 µL). 48 h after polymers addition, the cell culture medium was removed from each well and replaced with RPMI without serum (200 µL). (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MTT, Invitrogen) solution in PBS (5 mg/mL, 20 µL) was then added to obtain a final concentration of 0.5 mg MTT/mL. After 4 h incubation at 37°C, excess reagent was removed by aspiration. The formazan crystals were dissolved in DMSO (100 µL) and measured spectrophotometrically in a microplate reader at a wavelength of 570 nm. The relative cell viability of treated cells was calculated compared to untreated ones.

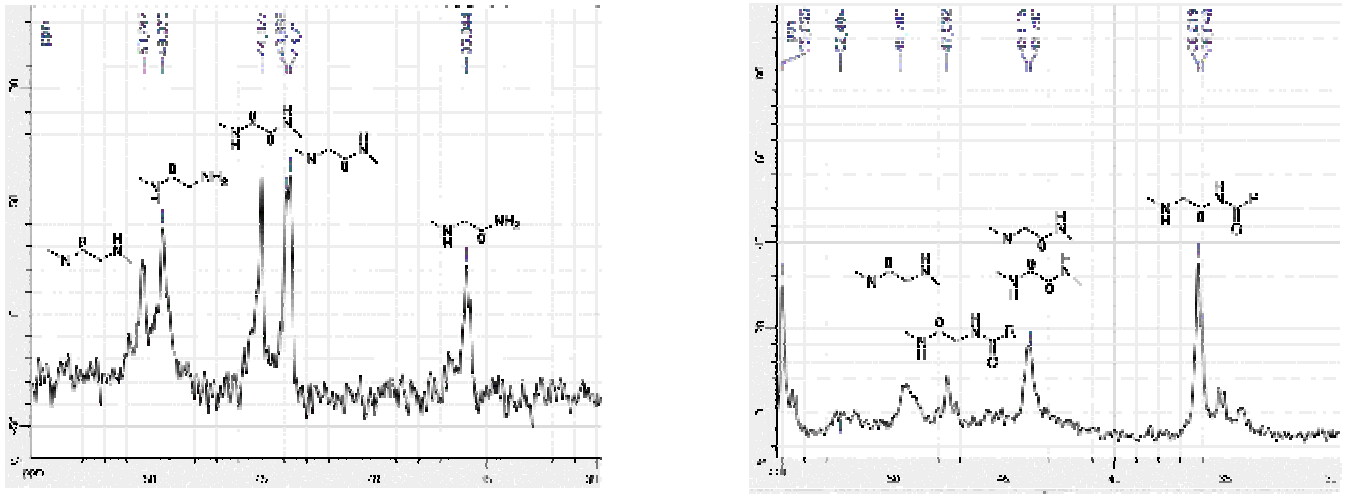

Figure S1. ^{13}C NMR spectra of the ethylenimine signals of branched PEI 25K (left) and PEIY (right). Each polymer (HCl form) was dissolved in D_2O. These data provide evidence of almost full modification of the PEI primary amines with tyrosine.

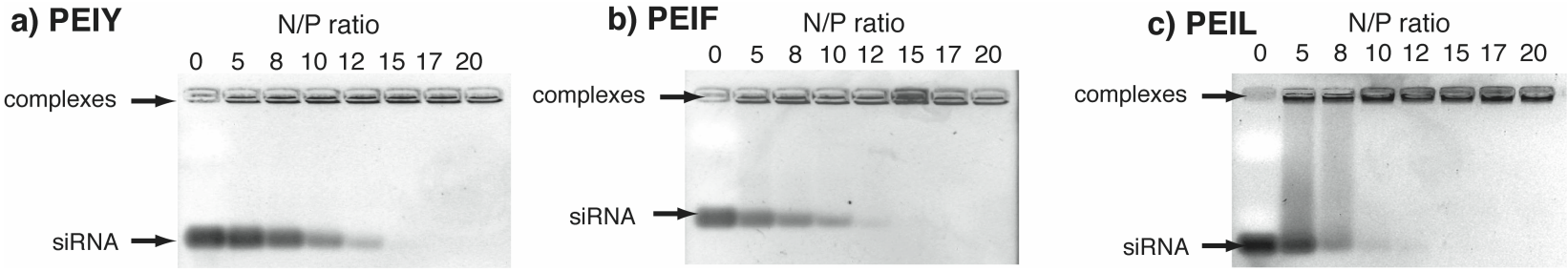

Figure S2. Complexation of siRNA by PEIY (a), PEIF (b), PEIL (c) as visualized by gel electrophoresis. Agarose gels were stained with ethidium bromide to visualize siRNA. Full Complexation of siRNA by the polymer intervened at the same N/P ratio of 15, for PEIY and PEIF, suggesting that hydroxyl group did not interfere dramatically with siRNA binding.

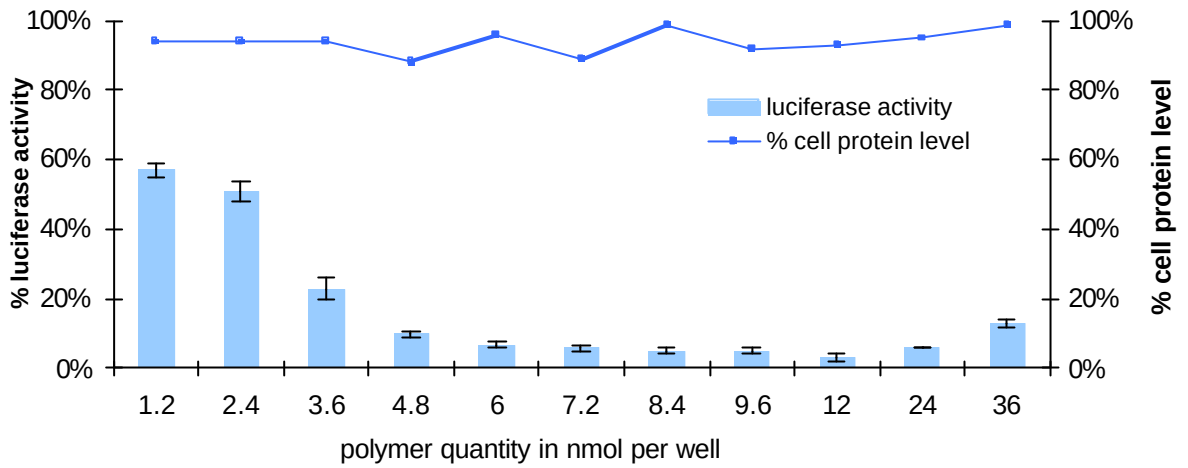

Figure S3. Evaluation of the siRNA delivery efficacy as a function of the PEIY polymer quantity.

Experimental conditions: The polymer was mixed with siRNAs (6 pmol) in 0.1 mL RPMI and added to A549-Luc cells grown in 0.5 mL medium containing serum. Final concentrations of siRNAs were 10 nM. Luciferase expression was measured 48 h after addition of the polyplexes. The bar represents the residual luciferase activity. The points measured the percentage of remaining cell proteins levels relative to mock. The shape of the figure is likely a combination of cell receptors (HSPGs) saturation by the cationic particles and dilution of the siRNA onto un-internalized PEIY self-assemblies.

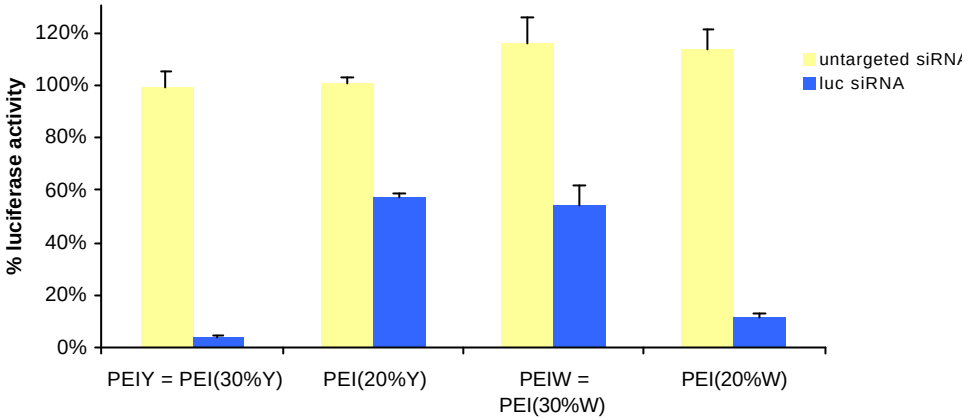

Figure S4. Effect of the amino acids content on the delivery efficacy. Decreasing the content of tyrosine of PEIY (30% tyrosine/ethylenimine unit) to 20% decreased the siRNA silencing efficacy. An opposite trend was observed for the more hydrophobic PEIW that led to better siRNA delivery with a lower tryptophane content. This experiment suggests that the efficacy of the polymer is due to a supramolecular hydrophobic/hydrophilic balance. Experimental conditions: Each polymer (12 nmol in ethylenimine) were mixed with untargeted (yellow bars) or luciferase (blue bars) siRNAs (6 pmol) in 0.1 mL RPMI and added to cells grown in 0.5 mL medium containing serum. Final concentrations of siRNAs were 10 nM and polymer concentration was 20 µM. Luciferase expression was measured 48h after addition of the polyplexes.

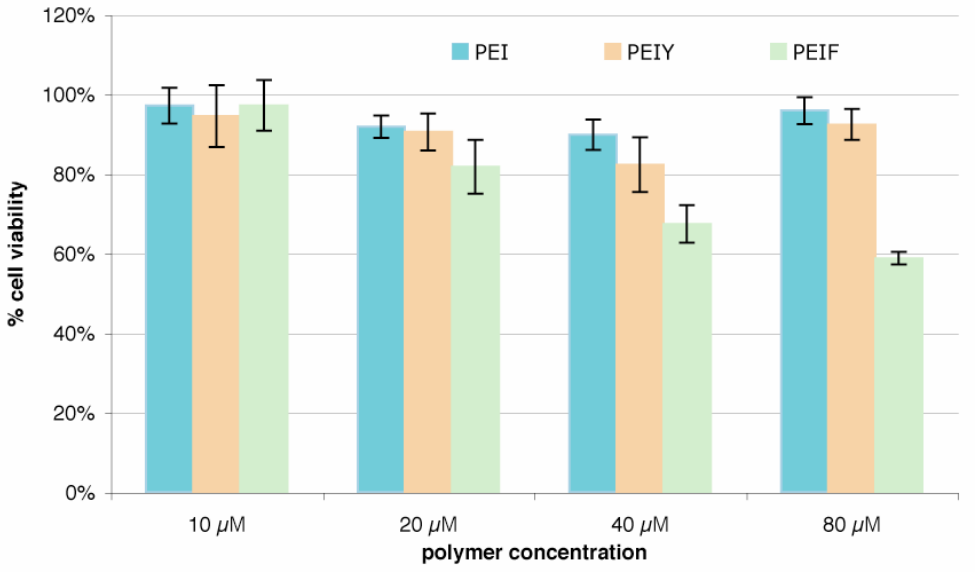

Figure S5. Estimation of A-549 cell viability in the presence of increasing concentrations of PEI, PEIY and PEIF. Cell viability was estimated by measuring the redox activity of living cells using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. PEI and PEIY did not modify the cell viability at concentration up to 80 µM whereas the more hydrophobic PEIF induced cell toxicity at 40 µM.

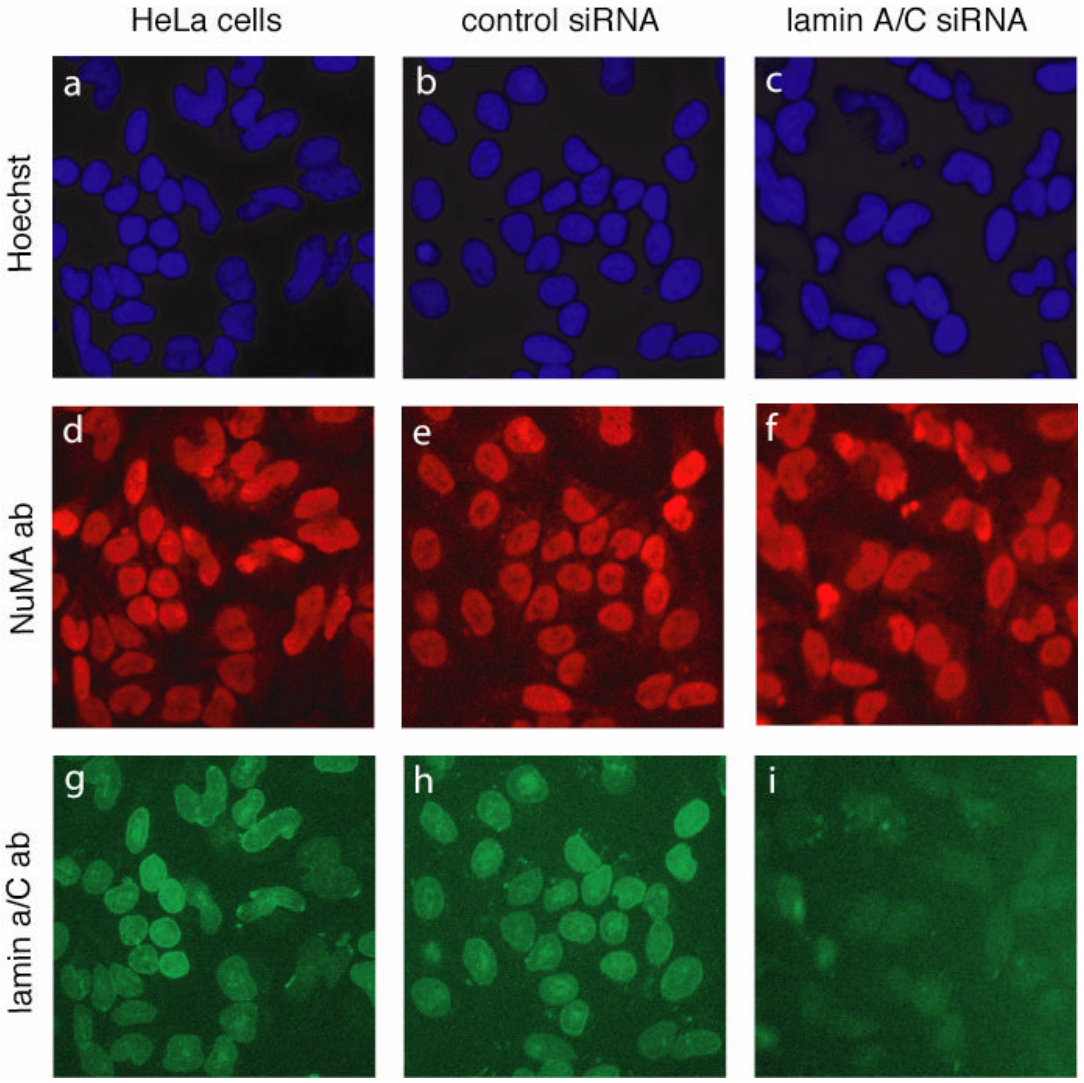

Figure S6. Silencing of nuclear envelope proteins lamin A/C in HeLa cells. Triple fluorescence staining [Hoechst staining of nuclear chromatin (a-b-c), NuMA specific antibody staining (d-e-f) and lamin A/C specific antibody staining (g-h-i)] of untransfected cells (a-d-g), cells transfected with 10 nM control siRNA (b-e-h) and with 10 nM specific siRNA (c-f-i).

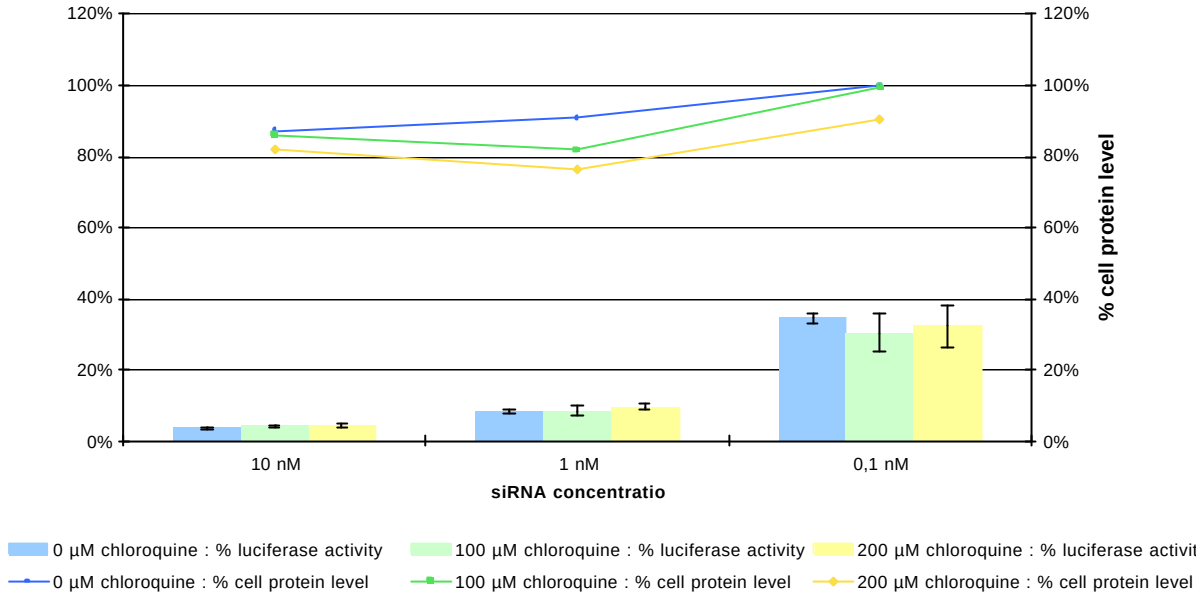

Figure S7. Chloroquine, an endosome disrupting agent did not alter/improve the efficacy of PEIY to deliver siRNA in cells: The polymer was mixed with siRNAs (6 pmol) in 0.1 mL RPMI and added to A549-Luc cells grown in 0.5 mL medium containing serum and 0, 100 or 200 µM chloroquine. After 4h incubation, the medium was replaced with a fresh one according to a described procedure.[3] Final concentrations of siRNAs were 10 nM. Luciferase expression was measured 48h after addition of the polyplexes. The bar represents the residual luciferase activity. The points measured the percentage of remaining cell proteins levels relative to cells grown in the presence of the same chloroquine concentration. Total Cell and protein amounts in the samples treated with 200 µM chloroquine were reduced to 40% relative to samples treated with 0 or 100 µM chloroquine.

References

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[2] Y.-H.Wang, Y.-W Hou, H.-J. Lee, *J. Biochem. Biophys. Methods* **2007**, *70*, 579-586

[3] P. Erbacher, A. C. Roche, M. Monsigny, P. Midoux, *Exp Cell Res* **1996**, *225*, 186.