Supplementary Information

PEG stabilized DNA - Organometallic Polymer Polyplexes for Gene

Delivery

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Experimental Part

Materials. Trimethylamine solution (31-35 wt%, 4.2 M in ethanol), potassium iodide (99%), iodoethane (99%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES), deoxyribonucleic acid sodium salt from salmon testes, 3-mercapto-1-propanesulfonic acid sodium salt (90%), Bovine serum albumin (\geq 99%), FBS, DMEM, penicillin, streptomycin sulfate, amphotericin B, trypsin/EDTA solution 10×, dicyclohexano-18-crown-6 (98%), 15-crown-5 (98%), H₂PtCl₆·6H₂O, DMSO-d₆ (99.9 atom% D), and toluene-d₈ (\geq 99.6 atom% D) were obtained from Aldrich and used as received. CDCl₃ (99.8 atom% D) was purchased from Cambridge Isotope Laboratories. pCMV-GFP plasmid DNA was obtained from PlasmidFactory GmbH & Co. KG, Bielefeld, Germany. O-[2-(3-Succinylamino)ethyl]-O'methyl-polyethylene glycol (PEG average $M_n = 750$ g/mol) was purchased from Rapp Polymere GmbH (Tübingen, Germany). Methanol, DMSO, THF and diethyl ether were obtained from Biosolve, the Netherlands. Milli-Q water (Millipore) was used in all experiments. Linear PEI (25 kDa) was purchased from Polysciences and CellTiter 96® One Solution Cell Proliferation Assay (MTS) was purchased at Promega, Madison, WI.

Techniques. ¹H and ¹³C NMR spectra were recorded on a Bruker Avance III 400 MHz instrument at 400.1 and 100.6 MHz, respectively, in CDCl₃, DMSO-d₆ or toluene-d₈. ¹H and ¹³C chemical shifts were based on the solvent residual signals. GPC measurements were carried out in THF (flow rate 2.0 mL/min) at 25 °C, using microstyragel columns (bead size

10 μ m) with pore sizes of 10⁶, 10⁵, 10⁴ and 10³ Å (Waters) and a dual detection system consisting of a differential refractometer (Waters model 410) and a differential viscometer (Viscotek model H502). Molar masses were determined relative to narrow polystyrene standards. FTIR spectra were measured with a Bruker ALPHA on freeze-dried samples.

The hydrodynamic particle size was measured with a Zetasizer Nano ZS (Malvern, Worcestershire, United Kingdom). This device uses a 4 mW 633 nm laser and measures the backscattering at a 173° angle. A delay time of 10 minutes after sample insertion was used for each measurement. The measured sample concentration was ~5 mg/mL.

Surface Plasmon resonance (SPR) measurements were performed in a SPR setup in Kretschmann configuration.

For fluorescent imaging a Keyence BZ-9000E (Keyence, Osaka, Japan) device was used.

Poly(ferrocenylsilane) 1. PFS **1** was synthesized by Pt-catalyzed ring-opening polymerization of [1](3-chloropropyl)methylsilaferrocenophane (3.0 g, 9.85 mmol) in THF, followed by halogen exchange using KI.¹ PFS **1** was isolated by precipitation in MeOH and dried under vacuum. Overall yield: 2.65 g (68%) of amber product. ¹H NMR (toluene-d₈, δ , ppm): 0.52 (SiCH₃, s, 3H); 1.00 (1-CH₂, m, 2H); 1.82 (2-CH₂, m, 2H); 2.95 (3-CH₂I, m, 2H); 4.05 + 4.10 + 4.27 + 4.29 (Cp, m, 8H). ¹³C NMR (toluene-d₈, δ , ppm): -2.83 (SiCH₃); 12.02 (1-C); 18.52 (2-C); 29.32 (3-CH₂I); 70.50 (Cp-Si); 71.96 + 73.95 (Cp). GPC (THF): $M_n = 2.2 \cdot 10^4$ g/mol, $M_w = 4.5 \cdot 10^4$ g/mol, $M_w/M_n = 2.07$.

Poly(ferrocenylsilane) 2. For 10% PEGylated PFS, 46.6 mg O-[2-(3-Succinvlamino)ethyl]-O'-methyl-polyethylene glycol (0.051 mmol, $M_n = 750$ g/mol) was dissolved in methanol (10 mL). Sodium hydroxide (0.051 mmol) was added to the flask and the reaction was continued at room temperature overnight. Methanol was evaporated and the formed salt was dissolved in DMSO (3 mL). PFS 1 (200 mg, 0.51 mmol r.u.) was dissolved in THF (10 mL) and the solution of PEG salt was added to the mixture. 15-Crown-5 (10 µl, 0.051 mmol) was added and the reaction was continued at 45 °C for 4 days. The same procedure was used for PFS with 25% of PEG side chains. ¹H NMR (CDCl₃, δ , ppm): 0.47 (SiCH₃, s, 3H); 0.87 + 1.01 (1-CH₂, m, 2H); 1.67 + 1.88 (2-CH₂, m, 2H); 2.48 (O-C=O-CH₂, m, 2H); 2.64 (N-C=O-CH₂, m, 2H); 3.22 + 4.04 (3-CH₂, m, 2H); 3.37 (MeO, s, 3H); 3.43 (N-CH₂, m, 2H); 3.64 (PEG, m) 4.00 + 4.23 (Cp, m, 8H). ¹³C NMR* (CDCl₃, δ , ppm): -2.98 (SiCH₃); 12.43 + 18.32 (1-C); 23.45 + 28.85 (2-C); 30.79 (O-C=O-CH₂, m, 2H); 29.53 (N-C=O-CH₂, m, 2H); 67.33 (3-CH₂, m, 2H); 59.18 (MeO, s, 3H); 39.32 (N-CH₂, m, 2H); 70.59 (PEG, m) 71.59 + 73.41 (Cp). *Based on C-H correlated NMR. ATR-FTIR (cm⁻¹): 1700 (carbonyl, PEG), 1111 (PEG), 1035 and 774 (PFS).

Poly(ferrocenylsilane) 3. PFS **1** (1.0 g, 2.53 mmol r.u.) was dissolved in THF (20 mL). Trimethylamine solution (10 mL, 4.2 M in ethanol) and DMSO (6.5 mL) were added to the solution. After 24 hours at room temperature, the product had precipitated in the reaction flask. THF was evaporated and fresh DMSO (10 mL) and trimethylamine (5 mL, 21 mmol) were added to the precipitate. After 3 days, the product was precipitated in THF. An orange solid was obtained (0.90 g, 2.03 mmol, 80%). PFS **3** was obtained through ion exchange by dialysis against 0.1 M aqueous NaCl for 1.5 day continuing with 1.5 day dialysis against Milli-Q water, followed by freeze-drying yielding the product as an amber foam with typical yields around 70%. The same procedure was used for PFS with PEG side chains (PFS **2**). ¹H NMR (DMSO-d₆, δ , ppm): 0.44 + 0.52 (SiCH₃, s, 3H); 0.83 (1-CH₂, m, 2H); 1.52 + 1.74 (2-CH₂, m, 2H); 2.13 (O-C=O-CH₂, m, 2H); 2.19 (N-C=O-CH₂, m, 2H); 3.10 (N-CH₃ s, 9H); 3.15 (N-CH₂, m, 2H); 3.23 (MeO, s, 3H); 3.39 + 3.40 (3-CH₂, m, 2H); 3.50 (PEG, m) 4.00-4.23 (Cp, m, 8H).

¹³C NMR (DMSO-d₆, δ , ppm): -3.38 (SiCH₃); 11.90 (1-C); 17.28 + 27.34 (2-C); 30.79* (O-C=O-CH₂, m, 2H); 29.53* (N-C=O-CH₂, m, 2H); 38.37 (N-CH₂); 51.98 (N-CH₃); 58.02 (MeO); 63.81 + 67.63 (3-CH₂); 69.22-70.42 (PEG) 70.9-71.4 + 73.09 (Cp) 177.56 + 178.20 (C=O). *Not observed anymore. ATR-FTIR (cm⁻¹): 1632 and 1479 (-NMe₃Cl).

Polyplex formation. Polymer/DNA complexes were prepared in variety of amine/phosphate (N/P) ratios ranging from 1 to 8 in 5.0 mM HEPES buffer solution at pH 7.4, vortex mixed for 5 seconds and incubated at room temperature for 30 minutes.²

Agarose gel retardation assay. The polyplexes at different N/P ratios were made by adding 10 μ L of polymer solution (varying concentrations in 10 mM HEPES, pH 7.4) to 10 μ L of DNA solution (80 μ g/mL in 10 mM HEPES), followed by vortexing for 5 s. The dispersions were incubated for 30 min at room temperature. After the addition of 3 μ L of 6× loading Dye (Thermo), 20 μ L of this mixture was applied onto a 1% agarose gel containing Midori Green Advance DNA stain and run at 100 V for 30 min, DNA was visualized under a Gel DocTM XR+ system (BioRad Laboratories Inc., Hercules, CA) with Image Lab software.

Protein adsorption. The SPR gold discs (gold thickness was 45 nm, XanTec Bioanalytics GmbH, Dusseldorf, Germany) were, after cleaning with piranha, functionalized with sodium 3-mercapto-1-propanesulfonate (1.0 mM in Milli-Q water). To measure the protein

adsorption on the polyplex particles, the substrate was mounted in the SPR setup and polyplex particles (100 μ g/mL in 5.0 mM HEPES buffer) were first adsorbed on the sulfonate modified substrate. Excess particles were washed away with buffer, after which a bovine serum albumin (BSA) solution (100 μ g/mL in 5.0 mM HEPES buffer) was passed over the substrate.

Cell culture. HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin and streptomycin (GIBCO) at 37 °C in a humidified 5% CO₂-containing atmosphere.

Transfection experiments. Transfection experiments were performed using 150 ng/mL of pCMV-GFP reporter plasmid. The cells were plated in 96-well plates (0.8-1.0 \cdot 10⁴ cells/well) and cultured in 0.1 mL/well DMEM medium containing 10% FBS for at least 24 h until 60-70% cell confluence was reached. They were then washed once with fresh PBS buffer and incubated in the DMEM medium with 0%, 5% or 10% FBS for transfection. The cells were incubated with 10 µL of the polyplex dispersion, added to the 0.1 mL medium, for 1 h at 37 °C in a 5% CO₂-containing atmosphere. The final concentrations of the PFS-Cation, PFS-Cation - 10% PEG and PFS-Cation - 25% PEG polyplexes, prepared at N/P 6, were 8.96, 10.83 and 13.66 µg/ml. For linear PEI at N/P 8, the concentration was 1.56 µg/mL. Linear PEI is non-cytotoxic at concentrations below 5 µg/mL. Next, the medium was replaced with DMEM medium containing 10% FBS and the cells were further incubated for another 47 h. All the transfection experiments were done in quadruplicate. A transfection formulation with linear PEI (25 kDa) prepared at an optimal N/P ratio of 8/1 was also applied as positive control.

Cell viability assay. Relative cell viability was evaluated by CellTiter 96[®] One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI). In brief, 20 μ l MTS was added to each well, including untreated cells (cells not exposed to polyplexes). After 4 hours in culture, which corresponds with the transfection time, the cell viability was determined by measuring the absorbance at 490 nm using a 550 BioRad plate-reader (Bio-Rad, Hertfordshire, UK).

Transfection efficiency assay. GFP gene expression in transfected cells was determined by quantification of the fluorescence intensity of GFP. After transfection, the cells in a 96-well plate were washed twice with PBS buffer and incubated with cell lysis buffer (100 μ L) at 4 °C for 20 min. The cell lysates were collected, centrifuged to pellet cellular debris, and 90 μ L of cell lysate was transferred to a 96-well black plate to determine fluorescence intensity of GFP with excitation and emission wavelengths of 488 nm and 520 nm, respectively.

Background fluorescence/auto-fluorescence was also determined using untreated cells as a blank control. The total recovered protein concentration in the cell lysate from each well was estimated with a BAC kit. The amount of protein was normalized from a BSA standard curve. GFP gene expression was calculated as fluorescence intensity (FI) normalized against protein concentration and expressed as arbitrary units (a.u.)/mg protein.







Figure S1. 400 MHz ¹H NMR spectra (δ , ppm) of a) PFS **1** (Toluene-d₈), b) PFS **2** with 25% PEG (CDCl₃), and c) the PFS-NMe₃Cl polymer (PFS **3**) with 25% PEG, used for the transfection studies (DMSO-d₆).



Figure S2. ¹³C NMR spectrum (DMSO-d₆, δ , ppm) of the PFS-NMe₃Cl polymer (PFS **3**) with 25% PEG, used for the transfection studies.



Figure S3. FTIR spectra of the PFS polymers used for the transfection studies. a) Full spectrum, b) detailed spectrum at $1750-500 \text{ cm}^{-1}$.

	$M_{\rm n}$ (kg/mol)	$M_{ m w}$ (kg/mol)	$M_{ m w}/M_{ m n}$
PFS 1	22	45	2.07
PFS 2 , 10 mol% PEG	28	61	2.18
PFS 2 , 25 mol% PEG	35	74	2.13

Table S4. Molar mass characteristics of PFS 1 and of PFS 2 with 10% and 25% PEG.



Figure S5. Agarose gel retardation assays show the ability of PFS **3** to condense plasmid DNA above a N/P ratio of 2.

AFM measurements were performed on cleaned silicon substrates using a Multimode AFM with a Nanoscope V controller (Bruker, Santa Barbara, California, USA) in tapping mode using silicon nitride cantilevers with resonance frequency $f_0 = 50-80$ kHz and spring constant of k = 0.35 N/m (Bruker, Camarillo, California, USA).



Figure S6. AFM tapping mode image of polyplex particles on a silicon substrate (left), and the height profile of the line shown in the left image (right) (N/P=4).



Figure S7. Fluorescence microscopy images confirm the high transfection efficiency of PFS **3**.

References

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