Supporting Information for

"Dendronised Block Copolymers as Potential Vectors for Gene Transfection"

Tony J. Wigglesworth,[a] Francisco Teixeira Jr.,[b] Fabian Axthelm,[b] Sara Eisler,[a] Noemi S. Csaba,[c] Hans P. Merkle,[c] Wolfgang Meier[b] and François Diederich[*a]

[a] *Laboratorium für Organische Chemie, ETH-Zürich, 8093 Zürich, Switzerland. Fax: +41 44 632 1109; Tel: +41 44 632 2992; E-mail: diederich@org.chem.ethz.ch*

[b] *Department of Chemistry, University of Basel, 4056 Basel, Switzerland. E-mail: wolfgang.meier@unibas.ch*

[c] *Institut für Pharmazeutische Wissenschaften, ETH-Zürich, 8093 Zürich, Switzerland. E-mail: hmerkle@pharma.ethz.ch*

Cell Biological Studies

Cell Culture. HeLa cells, a human cervix epithelial adenocarcinoma cell line, were used throughout this study and were obtained from American Type Culture Collection ATCC (Rockville, MD, USA). Cell culture was performed under standard conditions in humidified atmosphere with 5% CO_2. For cultivation, trypsin-EDTA 0.25% (Gibco, Paisley, UK), phosphate buffered saline, pH 7.4 (PBS) (Gibco, Paisley, UK) and cell culturing flasks (75 cm^2) (TPP, Fischer Scientific, Wohlen, Switzerland) were utilised. Cells were used within 20 passages and cultured as exponentially growing subconfluent monolayers in Dulbecco's modified Eagles medium (DMEM) with GlutaMAX, sodium pyruvate, 4.5 $mg.cm^{-3}$ glucose (Gibco, Paisley, UK), supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria) and penicillin/streptomycin (100 $units.cm^{-3}$ and 100 $\mu g.cm^{-3}$ respectively) (Gibco, Paisley, UK).

Plasmid DNA amplification. Plasmid DNA (pDNA) encoding green fluorescent protein (GFP) was used throughout the study. pDNA was generated by cloning the GFP sequence into the VR1012 vector which carries a kanamycin-resistance gene. pDNA was incorporated in competent Escherichia coli XL1 Blue cells with tetracycline resistance (Stratagene, La Jolla, USA). Cells were plated on agar plates containing kanamycin and tetracycline. A single clone was picked after 20 h and incubated in 300 cm^3 LB (Luria-Bertani) medium (10 g tryptone, 5 g yeast extract, and 0.5 g NaCl in 1000 cm^3) containing tetracycline at 50 $\mu g.cm^{-3}$ and kanamycin at 50 $\mu g.cm^{-3}$ for 10 h before transfer into LB medium containing kanamycin only for an additional 20 h according to a standard protocol. pDNA was purified using a Quiagen Gigakit (Quiagen, Hilden, Germany) according to the manufacturer's instructions and re-suspended in 10 mM Tris buffer, pH 8.5 (Quiagen plasmid purification handbook,

September 2000). The quality of the plasmid was checked using an agarose gel with Smart Ladder (Eurogentec, Seraing, Belgium). In addition, the quantity was calculated by measuring the electronic absorption at 260 nm and 280 nm. Working pDNA solutions were prepared by diluting the stock solutions to 0.1 $mg.cm^{-3}$ in Tris-EDTA (TE) buffer, pH 7.4 (*BioChemika Ultra*, Fluka).

Complex Formation. Block copolymers and pDNA encoding for GFP were complexed by electrostatic interactions. A given quantity of the respective copolymer solution (1.0 $mg.cm^{-3}$, 5% MeOH in deionised water) was added to pDNA solution (0.1 $mg.cm^{-3}$ in TE buffer, pH 7.4) to obtain a dispersion of the complex with the respective charge excess (CE) ratio. The dimensionless CE ratio is defined as the number of positive charges on the copolymer divided by the number of negative charges present on pDNA. Based on an average molecular weight of 660 $g.mol^{-1}$ per base pair (bp), 1 μg of pDNA is assumed to carry 3 nmol negative charges. To ensure efficient mixing, the resulting dispersions containing copolymer/pDNA complexes were pipetted up and down, and incubated for 30 min at 37 °C to complete complex formation.

Transfection in HeLa cells. Transfection experiments were performed in HeLa cells. All experiments were performed in triplicate. Cells were seeded in 24-well plates (Falcon, BD Labware, Franklin Lakes, NJ, USA) at a density of 40 000 $cells.cm^{-2}$ and were used 24 h after seeding as exponentially growing subconfluent monolayers. Copolymer/pDNA complexes were made as described above. For transfection, the pre-formed copolymer/pDNA complex dispersions were diluted with serum free medium. The mixtures were added to the cells at a concentration of 4.0 μg pDNA/well and incubated at 37 °C. After 4 h, the complex dispersion was removed and replaced with medium containing 10% FBS and incubated for 24 h. The cells

were washed with PBS (0.1 cm^3) and trypsinised (0.1 cm^3) for 5 min in order to detach them from the surface. Trypsinisation was stopped by the addition of medium containing serum (0.4 cm^3). Cells were then transferred into FACS tubes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and stored on ice. Cells were analysed by fluorescence-activated cell-sorting (FACS) using a BD FacsCanto flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) within 2 h. The FACS data was evaluated using FlowJo software (Tree Star Inc., Medford, OR, USA). A total of 6 000 events per sample were counted. The number of cells with fluorescence intensity higher than untransfected control cells was used to calculate the percentage of transfected cells. Linear poly(ethylene imine) (PEI) (M_p = 25 000, Alfa Aesar) was used as a positive control and complexed with pDNA at CE = 10 according to the method described above (see complex formation). Lipofectamine 2000™ (LPF) (Invitrogen) was used as a positive control following the manufacturer's instructions. The results are presented as the percentage of living cells transfected and are summarised graphically in Figure S1.

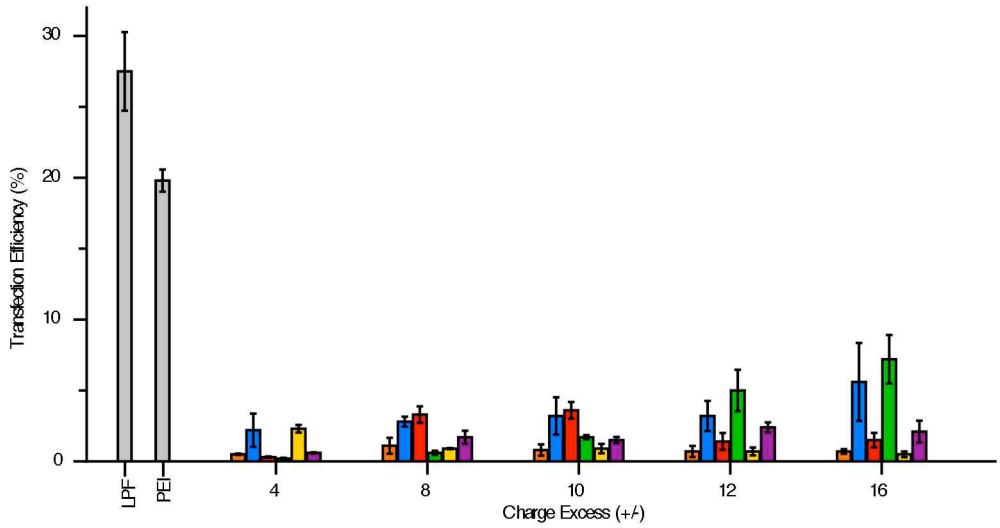

Figure S1 Transfection efficiency of dendronised block copolymers $\mathbf{G1_m}$-*b*-$\mathbf{PEG_n}$. Bars represent the percentage of living cells transfected after treatment with 4 μg of pDNA complexed with varying amounts of copolymers $\mathbf{G1_{100}}$ (orange), $\mathbf{G1_{20}}$-*b*-$\mathbf{PEG_{20}}$ (blue), $\mathbf{G1_{40}}$-*b*-$\mathbf{PEG_{40}}$ (red), $\mathbf{G1_{60}}$-*b*-$\mathbf{PEG_{60}}$ (green), $\mathbf{G1_{20}}$-*b*-$\mathbf{PEG_{10}}$ (yellow), and $\mathbf{G1_{60}}$-*b*-$\mathbf{PEG_{30}}$ (purple) at each respective CE in comparison with reference vectors LPF and PEI (grey).

Cell Viability. The viability of HeLa cells after incubation with the copolymer/pDNA complexes was evaluated by measuring the overall activity of mitochondrial dehydrogenase (MTT assay) according to the instructions of ATCC (MTT Cell Proliferation Assay Instructions). HeLa cells were seeded in 96-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 40 000 cells.cm^{-2} and were incubated for 24 h. Copolymer/pDNA complexes were added as described above at a concentration of 2 μg pDNA per well and incubated at 37 °C. After 4 h, the complex dispersions were removed, MTT reagent was added (0.1 cm^{-3}) (0.5 mg.cm^{-3} solution of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide in PBS (pH 7.4) and DMEM) and the cells were incubated at 37 °C. After 2 h, the MTT solution was removed and the purple precipitate in the cells was dissolved by the addition of detergent (0.1 cm^{-3}) (solution

of 81 cm^3 isopropanol, 15 cm^3 sodium dodecyl sulphate (SDS) (20 wt.-% aqueous solution), 4 cm^3 aqueous HCl solution (1 M)). After 16 h, at room temperature in the dark the overall activity of mitochondrial dehydrogenase in each well was measured spectrophotometrically at 570 nm using a ThermoMaz microplate reader (Molecular Devices, Sunnyvale, CA, USA). Untreated cells and cells treated with ethanol for 10 min prior to treatment with MTT were used as controls. The results are presented as the percentage of dead cells after treatment with copolymer/pDNA complexes under the transfection conditions and are summarised graphically in Figure S2.

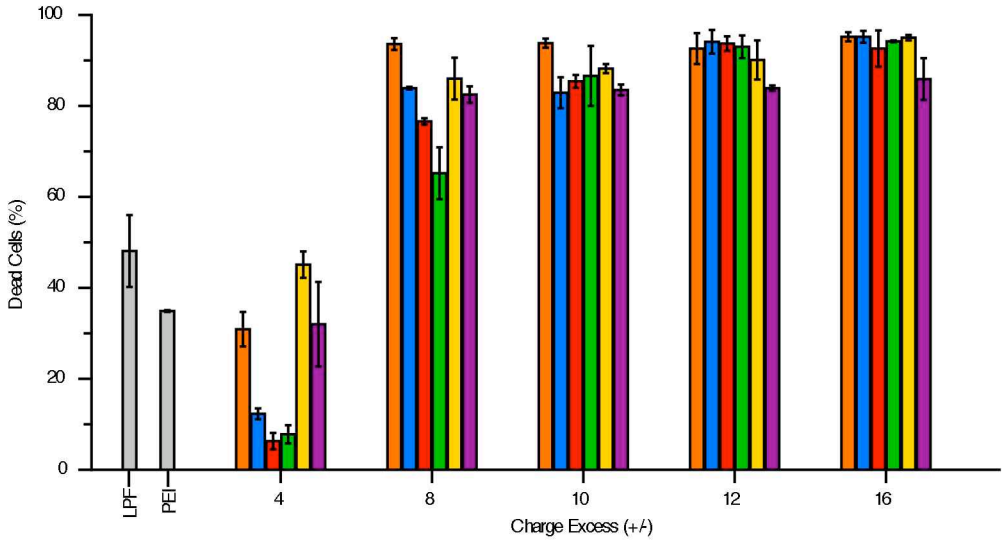

Figure S2 Toxicity of dendronised block copolymers $\mathbf{G1_m}$-*b*-$\mathbf{PEG_n}$. Bars represent the percentage of dead cells after treatment with 2 μg of pDNA complexed with varying amounts of copolymers $\mathbf{G1_{100}}$ (orange), $\mathbf{G1_{20}}$-*b*-$\mathbf{PEG_{20}}$ (blue), $\mathbf{G1_{40}}$-*b*-$\mathbf{PEG_{40}}$ (red), $\mathbf{G1_{60}}$-*b*-$\mathbf{PEG_{60}}$ (green), $\mathbf{G1_{20}}$-*b*-$\mathbf{PEG_{10}}$ (yellow), and $\mathbf{G1_{60}}$-*b*-$\mathbf{PEG_{30}}$ (purple) at each respective CE in comparison with reference vectors LPF and PEI (grey).

Analysis of copolymer/pDNA complexes.

Dynamic light scattering (DLS). Polyplex solutions containing 25 μg of pDNA and excess copolymer (CE = 2) were analysed by DLS as previously described in the manuscript. The calculated size distributions for each copolymer/pDNA complex are shown below in Figure S3.

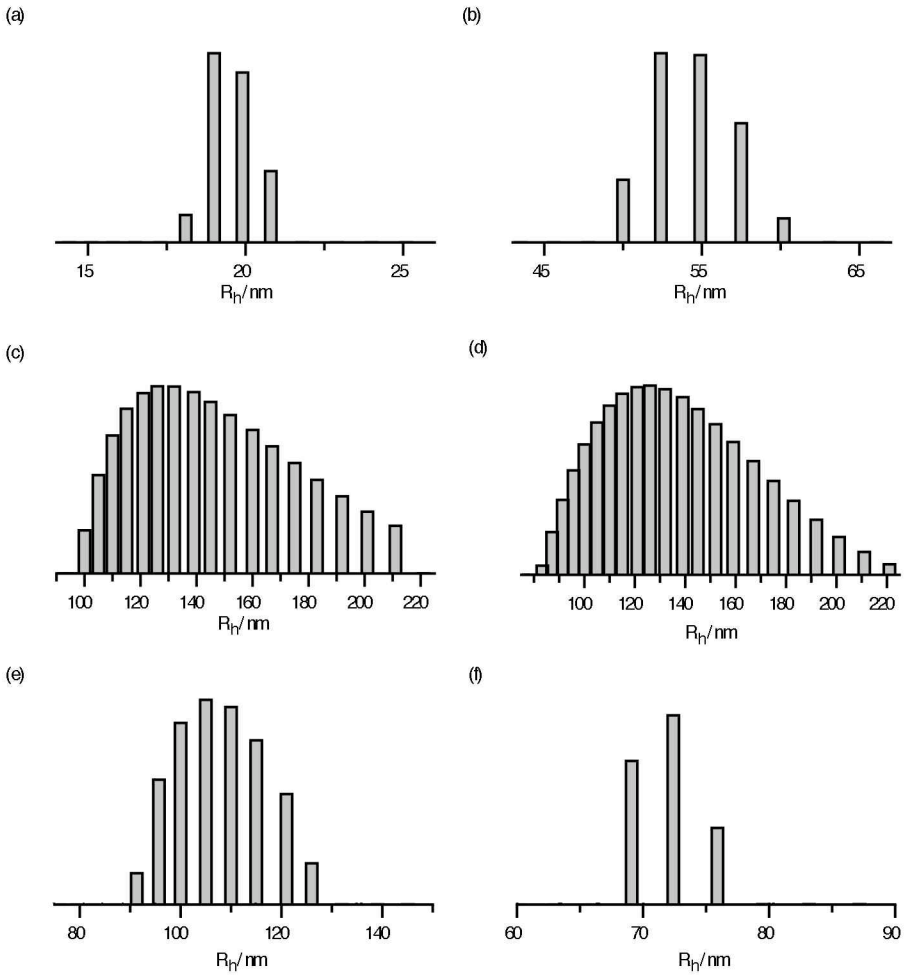

Figure S3 Size distribution of polyion complex micelles formed by complexing pDNA with block copolymers **G1$_m$-*b*-PEG$_n$** (CE = 2) at physiological pH (7.4). (a) **G1$_{100}$**/pDNA, (b) **G1$_{20}$-*b*-PEG$_{20}$**/pDNA, (c) **G1$_{40}$-*b*-PEG$_{40}$**/pDNA, (d) **G1$_{60}$-*b*-PEG$_{60}$**/pDNA, (e) **G1$_{60}$-*b*-PEG$_{30}$**/pDNA, (f) **G1$_{20}$-*b*-PEG$_{10}$**/pDNA.

Block copolymer solutions (0.5 mg.cm^{-3}) in TE buffer (pH 7.4) were analysed by DLS as previously described in the manuscript. The calculated size distributions for each block copolymer are shown below in Figure S4.

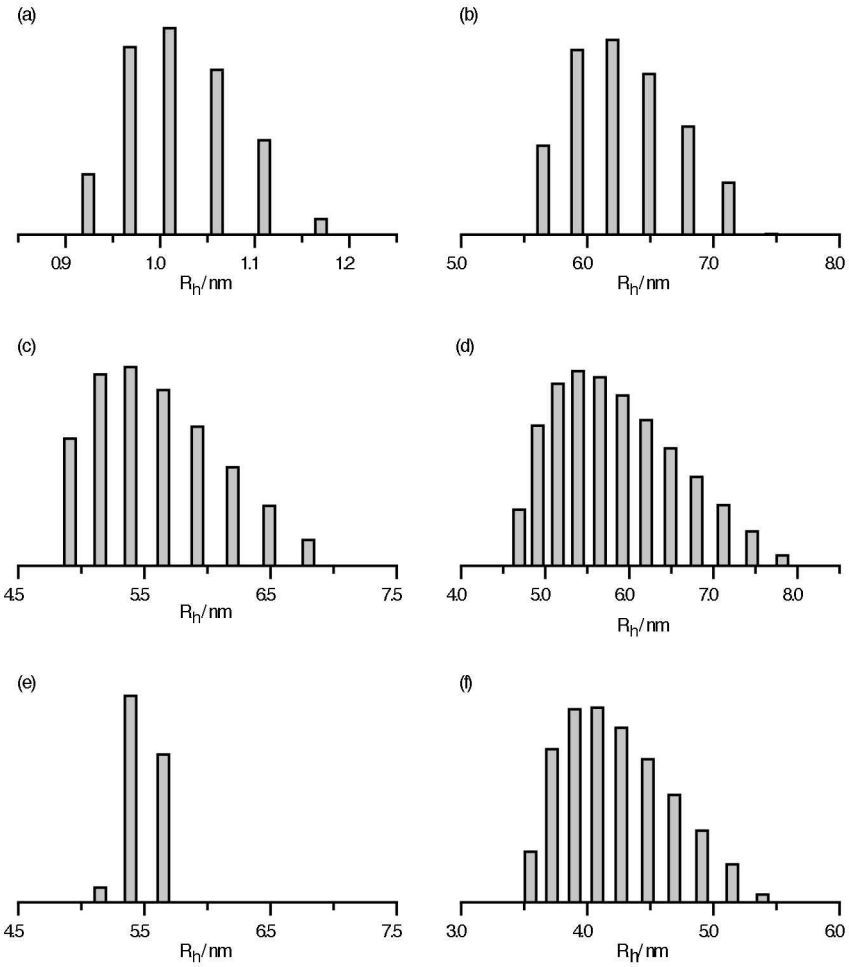

Figure S4 Size distribution of block copolymers **G1$_m$-*b*-PEG$_n$** (CE = 2) at physiological pH (7.4). (a) **G1$_{100}$,** (b) **G1$_{20}$-*b*-PEG$_{20}$**, (c) **G1$_{40}$-*b*-PEG$_{40}$**, (d) **G1$_{60}$-*b*-PEG$_{60}$**, (e) **G1$_{60}$-*b*-PEG$_{30}$**, (f) **G1$_{20}$-*b*-PEG$_{10}$**.

Transmission Electron Microscopy (TEM).

Polyplex solutions (CE = 2) were deposited on carbon coated grids and imaged by TEM as described in the manuscript. The resulting images are shown in Figure S5.

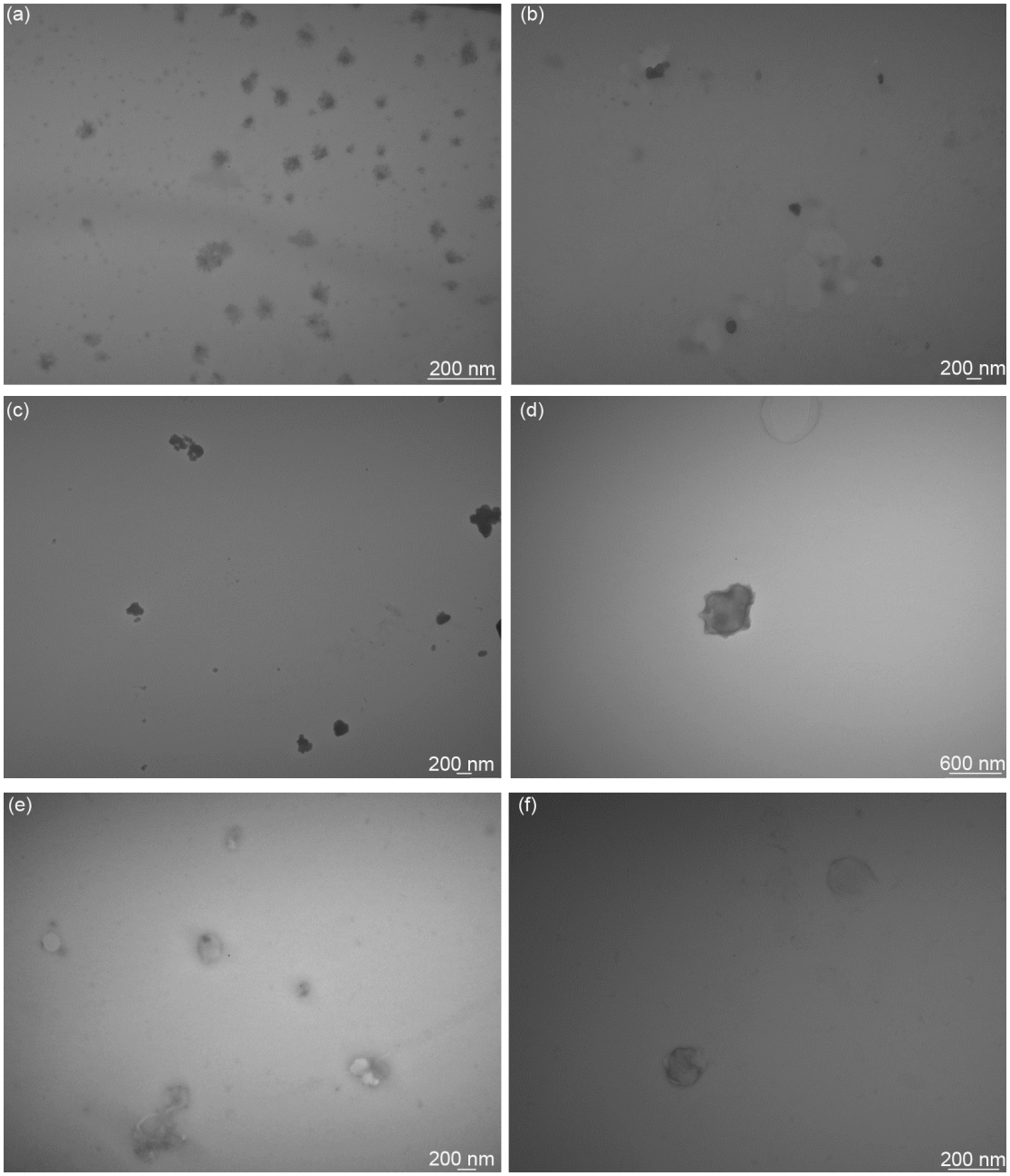

Figure S5 TEM images of copolymer/pDNA complexes at CE = 2. Complexes were drop-cast on carbon grids from aqueous solution (TE Buffer, pH 7.4) and stained with 1 wt.-% aqueous uranyl acetate solution. (a) $\mathbf{G1_{100}}$/pDNA, (b) $\mathbf{G1_{20}}$-***b***-$\mathbf{PEG_{20}}$/pDNA, (c) $\mathbf{G1_{40}}$-***b***-$\mathbf{PEG_{40}}$/pDNA, (d) $\mathbf{G1_{60}}$-***b***-$\mathbf{PEG_{60}}$/pDNA, (e) $\mathbf{G1_{60}}$-***b***-$\mathbf{PEG_{30}}$/pDNA, (f) $\mathbf{G1_{20}}$-***b***-$\mathbf{PEG_{10}}$/pDNA.

Polymer Synthesis and Characterisation

General procedures for the synthesis and deprotection of block copolymers **BocG1$_m$-*b*-PEG$_n$** are outlined in the manuscript. Experimental details, IR, ^{1}H NMR and ^{19}F NMR spectral characterisations of the copolymers are provided and representative ^{1}H NMR and ^{19}F NMR spectra are presented graphically in Figure S6 and Figure S7. The results of the GPC analysis are summarised in Table 1 in the manuscript and are not shown.

Block copolymer synthesis

BocG1$_{100}$

Monomer (±)-**M1** (0.12 g, 0.14 mmol) was polymerised with catalyst **6** (1.2 mg, 1.4 μmol) yielding homopolymer **BocG1$_{100}$** (0.11 g, 92%) as an off-white solid; δ_H($CDCl_3$, 300 MHz) 7.88 (s), 7.75 (s), 7.46 (br s), 5.47 (br s), 5.30 (br s), 5.03 (br s), 4.44 (br s), 3.40 (br s), 3.16 (br s), 2.47 (br s), 2.08 (br s), 1.87 (br s), 1.71 (br s) and 1.39 (br s, boc-CH_3).

BocG1$_{20}$-*b*-PEG$_{20}$

Monomers (±)-**M1** (0.20 g, 0.23 mmol) and (±)-**M2** (0.12 g, 0.23 mmol) were copolymerised with catalyst **6** (10.0 mg, 12 μmol) yielding block copolymer **BocG1$_{20}$-*b*-PEG$_{20}$** (0.27 g, 89%) as an off-white solid; δ_H($CDCl_3$, 300 MHz) 7.89 (br s), 7.76 (br s), 5.50 (br s), 5.29 (br s), 5.02 (br s), 4.54 (br s), 4.45 (br s), 3.65-3.64 (br m, PEG-OCH_2CH_2O), 3.41 (br s), 3.37 (s, PEG-OCH_3), 3.17 (br s), 2.79 (br, s), 2.47 (br s), 2.08 (br s), 1.89 (br, s), 1.73 (br s), 1.55 (br s) and 1.39 (br s, boc-CH_3).

BocG1$_{40}$-*b*-PEG$_{40}$

Monomers (±)-**M1** (0.20 g, 0.23 mmol) and (±)-**M2** (0.12 g, 0.23 mmol) were copolymerised with catalyst **6** (5.0 mg, 5.8 μmol) yielding block copolymer **BocG1$_{40}$-*b*-PEG$_{40}$** (0.27 g, 89%) as an off-white solid; δ_H($CDCl_3$, 300 MHz) 7.89 (br s), 7.76 (br s), 5.50 (br s), 5.29 (br s), 5.02 (br s), 4.54 (br s), 4.45 (br s), 3.65-3.64 (br m, PEG-OCH_2CH_2O), 3.41 (br s), 3.37 (s, PEG-OCH_3), 3.17 (br s), 2.79 (br, s), 2.47 (br s), 2.08 (br s), 1.89 (br, s), 1.73 (br s), 1.55 (br s) and 1.39 (br s, boc-CH_3).

BocG1$_{60}$-*b*-PEG$_{60}$

Monomers (±)-**M1** (0.20 g, 0.23 mmol) and (±)-**M2** (0.12 g, 0.23 mmol) were copolymerised with catalyst **6** (3.3 mg, 3.9 μmol) yielding block copolymer **BocG1$_{60}$-*b*-PEG$_{60}$** (0.28 g, 92%) as an off-white solid; δ_H($CDCl_3$, 300 MHz) 7.89 (br s), 7.76 (br s), 5.50 (br s), 5.29 (br s), 5.02 (br s), 4.54 (br s), 4.45 (br s), 3.65-3.64 (br m, PEG-OCH_2CH_2O), 3.41 (br s), 3.37 (s, PEG-OCH_3), 3.17 (br s), 2.79 (br, s), 2.47 (br s), 2.08 (br s), 1.89 (br, s), 1.73 (br s), 1.55 (br s) and 1.39 (br s, boc-CH_3).

BocG1$_{60}$-*b*-PEG$_{30}$

Monomers (±)-**M1** (0.20 g, 0.23 mmol) and (±)-**M2** (0.06 g, 0.11 mmol) were copolymerised with catalyst **6** (3.2 mg, 3.8 μmol) yielding block copolymer **BocG1$_{60}$-*b*-PEG$_{30}$** (0.24 g, 92%) as an off-white solid; δ_H($CDCl_3$, 300 MHz) 7.89 (br s), 7.76 (br s), 5.50 (br s), 5.29 (br s), 5.02 (br s), 4.54 (br s), 4.45 (br s), 3.65-3.64 (br m, PEG-OCH_2CH_2O), 3.41 (br s), 3.37 (s, PEG-OCH_3), 3.17 (br s), 2.79 (br, s), 2.47 (br s), 2.08 (br s), 1.89 (br, s), 1.73 (br s), 1.55 (br s) and 1.39 (br s, boc-CH_3).

BocG1$_{20}$-*b*-PEG$_{10}$

Monomers (±)-**M1** (0.20 g, 0.23 mmol) and (±)-**M2** (0.06 g, 0.11 mmol) were copolymerised with catalyst **6** (9.7 mg, 11 μmol) yielding block copolymer **BocG1$_{20}$-*b*-PEG$_{10}$** (0.24 g, 92%) as an off-white solid; δ_H($CDCl_3$, 300 MHz) 7.89 (br s), 7.76 (br s), 5.50 (br s), 5.29 (br s), 5.02 (br s), 4.54 (br s), 4.45 (br s), 3.65-3.64 (br m, PEG-OCH_2CH_2O), 3.41 (br s), 3.37 (s, PEG-OCH_3), 3.17 (br s), 2.79 (br, s), 2.47 (br s), 2.08 (br s), 1.89 (br, s), 1.73 (br s), 1.55 (br s) and 1.39 (br s, boc-CH_3).

Block copolymer deprotections

G1$_{100}$

Polymer **BocG1$_{100}$** (0.06 g) was deprotected with TFA yielding polymer **G1$_{100}$** (0.05 g, 99%) as an off-white solid; ν_{max}/cm^{-1} = 2934m, 1668s, 1538w, 1180s, 1129s, 840m, 798m and 722m; δ_H($(CD_3)_2SO$, 300MHz) 8.75 (br s), 7.98 (br s), 7.9 (br s), 7.3 (br s), 5.60 (br s), 5.30 (br s), 4.38 (br s), 2.82 (br s) and 2.11 (br s); δ_F($(CD_3)_2SO$, 376MHz) –74.1 (s, CF_3COO^-).

G1$_{20}$-*b*-PEG$_{20}$

Block copolymer **BocG1$_{20}$-*b*-PEG$_{20}$** (0.02 g) was deprotected with TFA yielding block copolymer **G1$_{20}$-*b*-PEG$_{20}$** (0.19 mg, 99%) as an off-white solid; ν_{max}/cm^{-1} = 2902s, 1674s, 1538w, 1199s, 1176s, 1124s, 1084s, 835m, 798m and 721m; δ_H($(CD_3)_2SO$, 300MHz) 8.76 (s), 7.98 (s), 7.90 (s), 7.32 (s), 7.22 (s), 5.61 (br s), 5.27 (br s), 4.41 (br s), 3.46 (br s, PEG-OCH_2CH_2O), 3.20 (s, PEG-OCH_3), 2.83 (br s), 2.12 (br s) and 1.77-1.63 (br m); δ_F($(CD_3)_2SO$, 376MHz) –73.9 (s, CF_3COO^-).

G1$_{40}$-*b*-PEG$_{40}$

Block copolymer **BocG1$_{40}$-*b*-PEG$_{40}$** (0.18 13) was deprotected with TFA yielding block copolymer **G1$_{40}$-*b*-PEG$_{40}$** (0.17 g, 99%) as an off-white solid; ν_{max}/cm^{-1} = 2866s, 1674s, 1537w, 1199s, 1176s, 1125s, 1096s, 835m, 798m and 721m; δ_H((CD$_3$)$_2$SO, 300MHz) 8.76 (s), 7.98 (s), 7.90 (s), 7.32 (s), 7.22 (s), 5.61 (br s), 5.27 (br s), 4.41 (br s), 3.46 (br s, PEG-OCH$_2$CH$_2$O), 3.20 (s, PEG-OCH$_3$), 2.83 (br s), 2.12 (br s) and 1.77-1.63 (br m); δ_F((CD$_3$)$_2$SO, 376MHz) –73.9 (s, CF$_3$COO$^-$).

G1$_{60}$-*b*-PEG$_{60}$

Block copolymer **BocG1$_{60}$-*b*-PEG$_{60}$** (0.18 g) was deprotected with TFA yielding block copolymer **G1$_{60}$-*b*-PEG$_{60}$** (0.17 g, 99%) as an off-white solid; ν_{max}/cm^{-1} = 2865s, 1674s, 1537w, 1199s, 1176s, 1122s, 1093s, 834m, 798m and 720m; δ_H((CD$_3$)$_2$SO, 300MHz) 8.76 (s), 7.98 (s), 7.90 (s), 7.32 (s), 7.22 (s), 5.61 (br s), 5.27 (br s), 4.41 (br s), 3.46 (br s, PEG-OCH$_2$CH$_2$O), 3.20 (s, PEG-OCH$_3$), 2.83 (br s), 2.12 (br s) and 1.77-1.63 (br m); δ_F((CD$_3$)$_2$SO, 376MHz) –73.8 (s, CF$_3$COO$^-$).

G1$_{60}$-*b*-PEG$_{30}$

Block copolymer **BocG1$_{60}$-*b*-PEG$_{30}$** (0.16 g) was deprotected with TFA yielding block copolymer **G1$_{60}$-*b*-PEG$_{30}$** (0.15 g, 99%) as an off-white solid; ν_{max}/cm^{-1} = 2867s, 1674s, 1537w, 1199s, 1177s, 1126s, 1094s, 835m, 798m and 721m; δ_H((CD$_3$)$_2$SO, 300MHz) 8.76 (s), 7.98 (s), 7.90 (s), 7.32 (s), 7.22 (s), 5.61 (br s), 5.27 (br s), 4.41 (br s), 3.20 (s, PEG-OCH$_3$), 2.83 (br s), 2.12 (br s) and 1.77-1.63 (br m); δ_F((CD$_3$)$_2$SO, 376MHz) –74.0 (s, CF$_3$COO$^-$).

G1$_{20}$-*b*-PEG$_{10}$

Block copolymer **BocG1$_{60}$-*b*-PEG$_{30}$** (0.17 g) was deprotected with TFA yielding block copolymer **G1$_{60}$-*b*-PEG$_{30}$** (0.16 g, 99%) as an off-white solid; ν_{max}/cm^{-1} = 2870s, 1674s, 1538w, 1200s, 1178s, 1127s, 1091s, 836m, 798m and 721m; $\delta_H((CD_3)_2SO, 300MHz)$ 8.76 (s), 7.98 (s), 7.90 (s), 7.32 (s), 7.22 (s), 5.61 (br s), 5.27 (br s), 4.41 (br s), 3.20 (s, PEG-OCH_3), 2.83 (br s), 2.12 (br s) and 1.77-1.63 (br m); $\delta_F((CD_3)_2SO, 376MHz)$ −73.9 (s, CF_3COO^-).

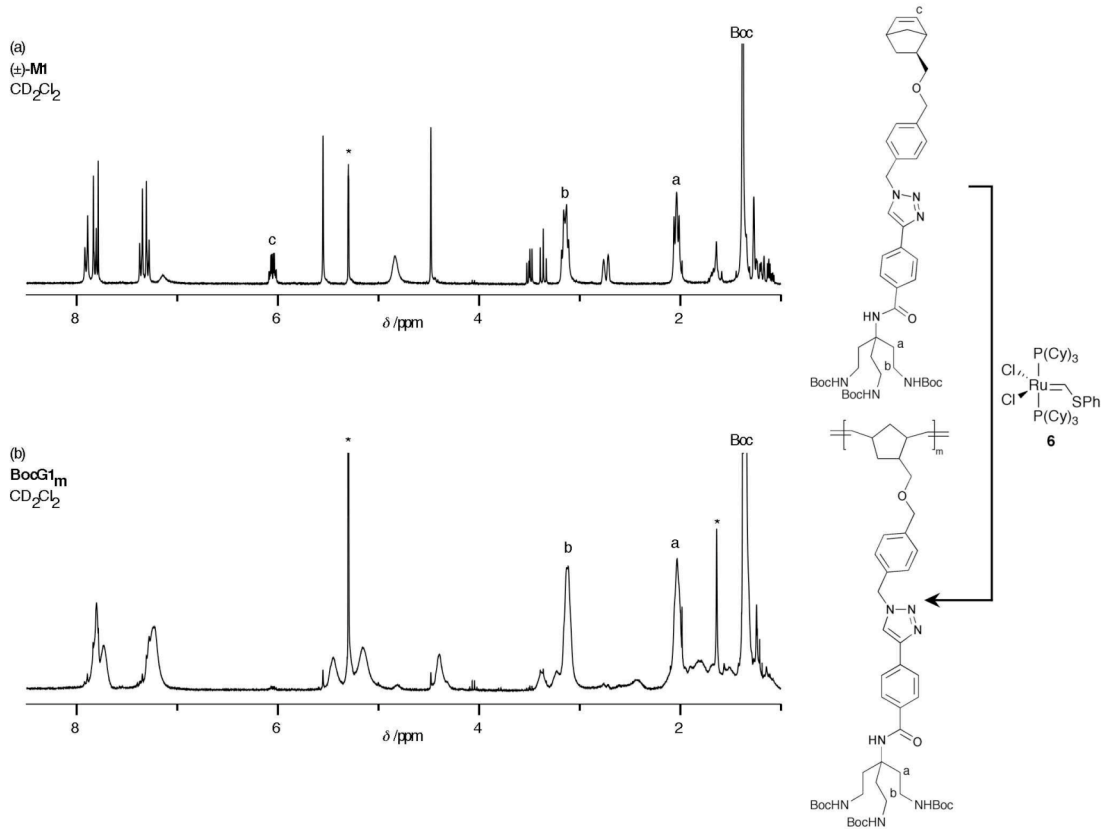

Figure S6 NMR scale polymerisation of monomer (±)-**M1** in CD_2Cl_2. ^1H NMR spectra of (a) the polymerisation reaction immediately after treatment with catalyst **6** and (b) after 90 min, monomer (±)-**M1** was completely converted into polymer **BocG1$_m$**. Polymer end groups were omitted for clarity. Solvent peaks are marked with an asterisk *.

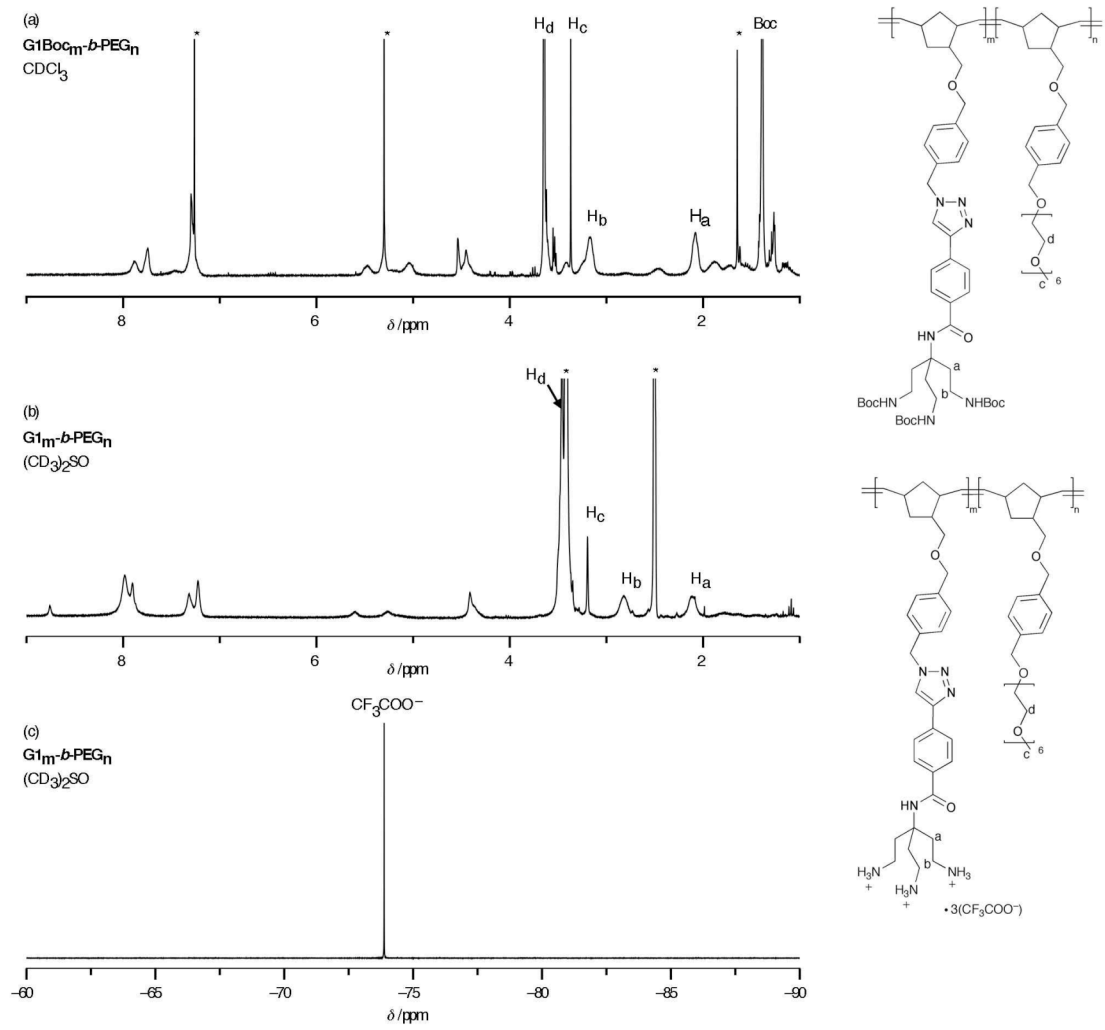

Figure S7 Representative ^{1}H NMR spectra of (a) **BocG1$_m$-*b*-PEG$_n$** in $CDCl_3$ and (b) **G1$_m$-*b*-PEG$_n$** in $(CD_3)_2SO$. (c) Representative ^{19}F NMR spectra of **G1$_m$-*b*-PEG$_n$** in $(CD_3)_2SO$. Polymer end groups were omitted for clarity. Solvent peaks are marked with an asterisk *.