Supporting information

Synthesis and application of spermine-based amphiphilic poly(β-amino ester)s for siRNA delivery

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1. Materials and Methods

1.1 Materials

Spermine (Fisher Scientific, Acros, USA), 1,4-butanediol diacrylate (TCI, Japan), chloroform D (Eurisotop, Germany), hyperbranched polyethylenimine (Lupasol® WF, BASF, Germany), n-hexane, dichloromethane (DCM), dimethyl sulfoxide (DMSO) were used as received unless otherwise stated.

Deuterium oxide, decylamine, HEPES (4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid), thiazolyl blue tetrazolium bromide (MTT), RPMI-1640 medium, fetal bovine serum (FBS), Penicilin-Streptomycin solution, Dubecco's Phosphate Buffered Saline (PBS), trypsin-EDTA solution (0.05%), and Geniticin (G418) disulfate solution were purchased from Sigma-Aldrich (Taufkirchen, Germany). SYBR Gold Dye, Lipofectamine™ 2000, AlexaFluor 488 (AF488) were bought from Life Technologies (Darmstadt, Germany). H1299 cells were purchased from ATCC (LG Promochem, Wesel, Germany), and H1299/eGFP cells were engineered as described before.[1]

Amine-modified eGFP siRNA (5´-pACCCUGAAGUUCAUCUGCACCACcg, 3´-ACUGGGACUUCAAGUAGACGGGUGGC) and scrambled siRNA (5´-pCGUUAAUCGCGUAUAAUACGCGUat, 3´-CAGCAAUUAGCGCAUAUUAUGCGCAUAp) were purchased from Integrated DNA Technologies (Leuven, Belgium). In the sequences, "p" represents a phosphate residue, lower case letters denote 2'-deoxyribonucleotides, capital letters express ribonucleotides, and underlined capital letters are 2'-O-methylribonucleotides.

NMR spectroscopy was performed on a Bruker AV500 spectrometer. Shifts were reported relative to the residual proton signal of the deuterated solvent. The size exclusion chromatographic assembly (SEC) running with chloroform (40 °C) contained a *PSS* SDV 5 µm 8*50 mm guard column with three *PSS* SDV 100,000 Å 5 µm 8*50 mm columns connected with a refractive index (RI) detector *Agilent* 1200 Series G1362A. Samples had concentrations of 1 mg/ml. Relative molar masses were determined using polystyrene standards.

1.2 Synthesis and characterization of amphiphilic poly(β-amino ester)s

1.2.1 Synthesis of tri-tert-butyl carbonyl spermine (tri-boc spermine)

Tri-tert-butyl carbonyl spermine abbreviated as tri-Boc-spermine was synthesized as described elsewhere with some modification.[2, 3] In brief, spermine (1 eq) was dissolved in methanol and stirred at -78 °C, ethyl trifluoroacetate (1 eq) was added dropwise subsequently and stirred at -78 °C for 1 h, then 0 °C for 1 h. Without isolation, di-tert-butyl dicarbonate (4 eq) was added

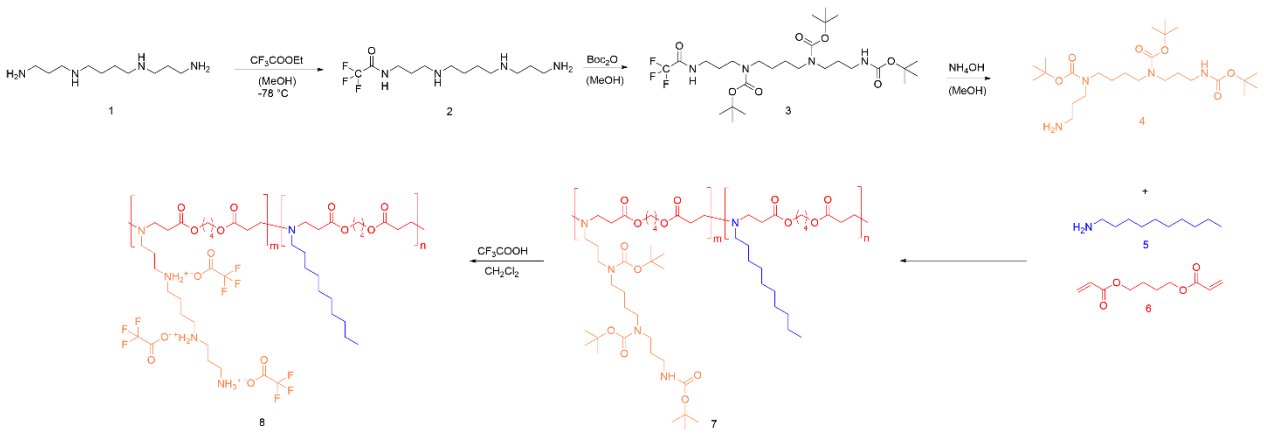
dropwise to the solution and stirred at room temperature for 2 days. Finally, the solution was adjusted to a pH above 11 by adding 25% ammonia in water and stirred overnight to cleave the trifluoroacetamide protecting group. The solvent of the mixture was then removed in vacuo and the residue was diluted with dichloromethane (DCM) and washed with distilled water and saturated NaCl aqueous solution. The combined organic phases were dried over $MgSO_4$, filtered and concentrated in vacuo to give the crude product. The crude product was purified by column chromatography (CH_2Cl_2\MeOH\NH_3, aq. 7:1:0.1, SiO_2, $KMnO_4$; R_f = 0.413) to yield tri-boc-spermine as a colorless liquid. Yield: 37%.

^{1}H NMR (400 MHz, CDCl3, 298 K): δ (ppm) = 1.43–1.48 (m, 31H, 6-CH_2, 7-CH_2, O–C–(CH_3)3×3, overlapping), 1.65 (m, 6H, 2-CH_2, 11-CH_2, NH_2), 2.68 (t, 2H, 12-CH_2), 3.08–3.23 (m, 10H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2).

1.2.2 Synthesis of P(BSpDBAE)

For the synthesis of tri-boc-spermine, decylamine, 1-4-butanediol diacrylate-based poly(tri-boc-spermine-co decylamine β-amino ester), abbreviated as P(BSpDBAE), tri-boc-spermine (1 eq), decylamine (1 eq) and 1,4-butanediol diacrylate (2 eq) were mixed and stirred at 120 ˚C overnight. The crude product was then dissolved in 2 mL DCM and precipitated in 50 mL n-hexanes at 4˚C. After centrifugation, the supernatant was discarded, and the product was dried in a vacuum oven for 2 days (50˚C, 15 mbar). The product P(BSpDBAE) was obtained as a brown semi-solid substance and characterized via ^{1}H NMR spectroscopy and size-exclusion chromatography (**Fig S4**, measurement relative to polystyrene and in chloroform at 40°C, 1 mg/mL). Yield: 44%.

^{1}H NMR (400 MHz, CDCl3, 298 K): δ (ppm) = 4.15 (d, 8H, 4CH_2), 2.99 (d, J = 142.2 Hz, 23H), 1.72 (d, J = 18.6 Hz, 15H), 1.55 – 1.38 (m, 20H), 1.27 (d, J = 12.7 Hz, 17H), 0.93 – 0.81 (m, 4H).



Scheme S1. Synthesis route of P(SpDBAE).

1.2.3 Synthesis of P(SpDBAE)

Deprotected P(SpDBAE) was synthesized by dissolving P(BSpDBAE) (130 mg) in 20 mL DCM and subsequently adding 1.2 mL TFA to the solution which was stirred at room temperature for 2 h. The solvents were then evaporated in vacuo and the residue was dissolved in DCM and precipitated from n-hexanes at 4°C. After centrifugation, the supernatant was discarded, and the product was freeze-dried from water and obtained as a yellow semi-solid substance. The product was characterized via ^{1}H NMR spectroscopy and named P(SpDBAE). Yield: 62%.

^{1}H NMR (500 MHz, D2O, 298 K): δ (ppm) = 4.16 (s, 8H), 3.54 (s, 6H), 3.34 (s, 2H), 3.12 (dt, *J* = 26.9, 8.1 Hz, 9H), 2.91 (d, *J* = 46.2 Hz, 6H), 2.23 (s, 1H), 2.08 (p, *J* = 8.3 Hz, 2H), 1.75 (d, *J* = 22.0 Hz, 15H), 1.26 (s, 21H), 0.85 (s, 5H).

1.3 Preparation and characterization of P(SpDBAE) polyplexes

To prepare P(SpDBAE) polyplexes (complexes of the polymer P(SpDBAE) and siRNA), P(SpDBAE) was first dissolved in water at a concentration of 1 mg/mL. The amount of polymer required for each formulation was then calculated according to the N/P ratio and aliquoted in 50 µL 10 mM HEPES buffer (pH 5.3). Subsequently, siRNA in 50 µL 10 mM HEPES buffer was mixed with the corresponding polymer solution and incubated at room temperature for 2 h. The size and Zeta potential of the polyplexes in 10 mM HEPES buffer were assessed with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

The amount of polymer was calculated according to the following equation:

$$M \text{ (polymer in pg)} = n \text{ siRNA (pmol)} \times 52 \times N/P \times 220 \text{ (g/mol)},$$

Where 52 is the number of nucleotides of the 25/27mer siRNA; N/P ratio is the molar ratio of the polymer protonable amines (N) and the siRNA phosphate groups (P); 220 g/mol is the protonable unit of P(SpDBAE). The protonable unit was calculated by dividing the molar mass of repeating unit by the number of protonable amines in each repeating unit. For P(SpDBAE): 220 g/mol = (Mw(decylamine)+Mw(spermine)+2xMw(1,4-butanediol diacrylate)+3xMw(TFA))/5 amines.

1.4 SYBR gold assay of P(SpDBAE) polyplexes

To determine the siRNA encapsulation efficiency of the polymer, SYBR gold assays were performed. In brief, the P(SpDBAE)-siRNA polyplex formulations from N/P 1 to 20 were prepared as described in section 1.3. Triplicates of 100 µL of the polyplexes were added to black 96-well plates, 30 µL 4X SYBR gold solution was added to each well subsequently, and the polyplexes were incubated with the intercalating dye at room temperature in the dark for 10 min. Finally, intercalation-based fluorescence was quantified using a microplate reader (TECAN, Switzerland, excitation: 485/20 nm, emission: 520/20 nm.). The fluorescence intensity of free siRNA (N/P = 0) was set as 100%.

1.5 Transmission electron microscopy of P(SpDBAE) polyplexes

The morphology of P(SpDBAE) polyplexes was visualized via transmission electron microscopy using an FEI Titan Themis 60-300 microscope (Thermo Fisher Scientific, Schwerte, Germany). In brief, P(SpDBAE) polyplexes were prepared at N/P 10 with 6.1 µM siRNA in 10 mM HEPES buffer. The polyplex suspension was deposited onto a copper grid, stained with 2% (w/v) phosphotungstic acid and finally imaged.

1.6 In vitro cell compatibility of P(SpDBAE) free polymer and P(SpDBAE) polyplexes

The *in vitro* cell compatibility of P(SpDBAE) free polymer and P(SpDBAE)/siRNA polyplexes were evaluated via MTT assay. In brief, H1299 cells were seeded in 96-well plates (5000 cells/well) 24 h

before use. P(SpDBAE) diluted in RPMI-1640 complete medium in a concentration range from 1 µg/mL to 100 µg/mL was added to the plate (100 µL per well in triplicates). After incubation in the CO_2 incubator for 24 h, the polymer solution was discarded and replaced with MTT solution in serum-free RPMI-1640 and incubated for 4 h in the incubator. Finally, the MTT solution was discarded, 100 µL per well DMSO was added and incubated at room temperature for 30 min. The optical density was finally determined at 570 nm and corrected with background measured at 680 nm using a microplate reader (TECAN, Switzerland).

For the MTT assay of P(SpDBAE) polyplexes, 10 µL polyplexes at N/P 5, 7 and 10 in 90 µL RPMI-1640 complete medium was incubated with H1299 cells the in CO_2 incubator for 24 h and treated with MTT solution and measured the same as described above. Hyperbranched polyethylenimine 25 kDa (hyPEI25K) and PEI polyplexes were used for comparison.

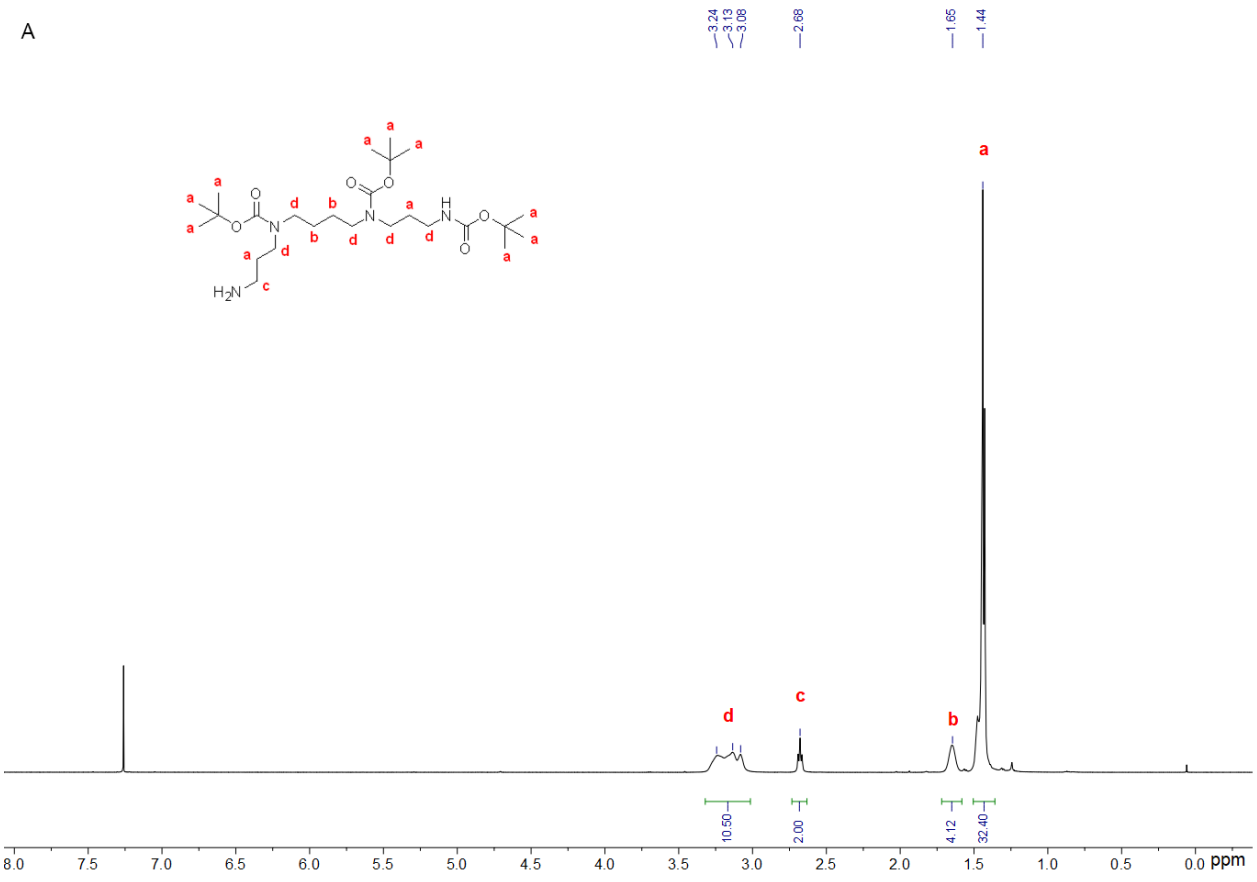
1.7 Cellular uptake of P(SpDBAE) polyplexes

The cellular uptake of P(SpDBAE) polyplexes was quantified by flow cytometry. The polyplexes were prepared as described in section 2.3 using Alexa Fluor 488 labeled siRNA (AF488-siRNA). Hyperbranched polyethylenimine 25 kDa (hyPEI25K) was used as control. H1299 cells were seeded in 24-well plates (50,000 cells in 1 mL medium/well). After incubation in the CO_2 incubator (37 °C, 5% CO_2) for 24 h, polyplexes at N/P 5, 7 and 10 in RPMI-1640 complete medium were added (50 pmol AF488-siRNA/well) and incubated in the incubator for 24 h. Subsequently, the polyplex suspension was discarded, the cells were rinsed with PBS and detached with 0.05% Trypsin-EDTA. The detached cells were then washed with PBS for another 2 times and analyzed via flow cytometry (Attune NxT Acoustic Focusing Cytometer, Darmstadt, Germany) with or without quenching by Trypan blue. A 488 nm laser for excitation and BL1 filter (530/30 nm) for detection.

1.8 GFP knockdown of P(SpDBAE) polyplexes

To determine the gene silencing ability of P(SpDBAE) polyplexes on the protein level, silencing of the enhanced green fluorescent protein reporter gene (eGFP) was quantified by flow cytometry. H1299/eGFP cells were seeded in 24-well plates (25000 cells in 500 µL medium/well), after growth in the CO_2 incubator (37˚C, 5% CO_2) for 24 h, the cells were transfected with

P(SpDBAE) polyplexes composed of scrambled siRNA (siNC, 50 pmol/well) or siRNA against GFP (siGFP, 50 pmol/well). Lipofectamine™ 2000 formulated with siNC and siGFP were respective control groups. For instance, 4 µL Lipofectamine 2000 was added to 196 µL OptiMEM medium and incubated at room temperature for 5 min. In parallel, 3 µL of siRNA (100 µM) was mixed with 297 µL OptiMEM medium and then 200 µL of the siRNA solution was pipetted to the Lipofectamine solution and incubated at room temperature for 20 min. In the next step, 100 µL of the lipofectamine:siRNA (lipoplexes) suspension was added to each well of the 24-well plate. After incubation in the incubator for 48 h, the cells were detached with 0.05% Trypsin-EDTA and washed with PBS for flow cytometry measurements (Attune Cytometer, Thermo Fisher Scientific, Darmstadt, Germany) with a 488 nm laser for excitation and 530/30 nm filter for detection.



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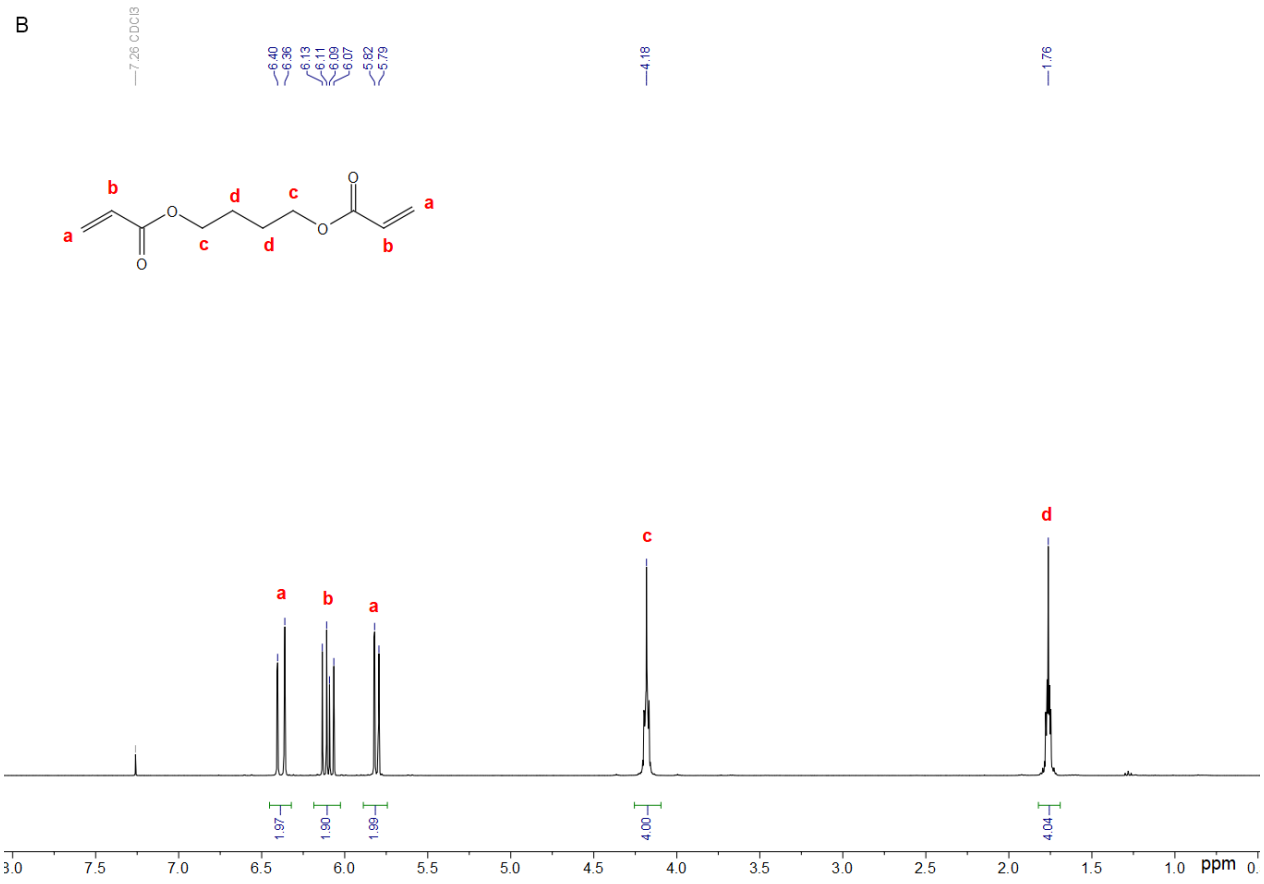
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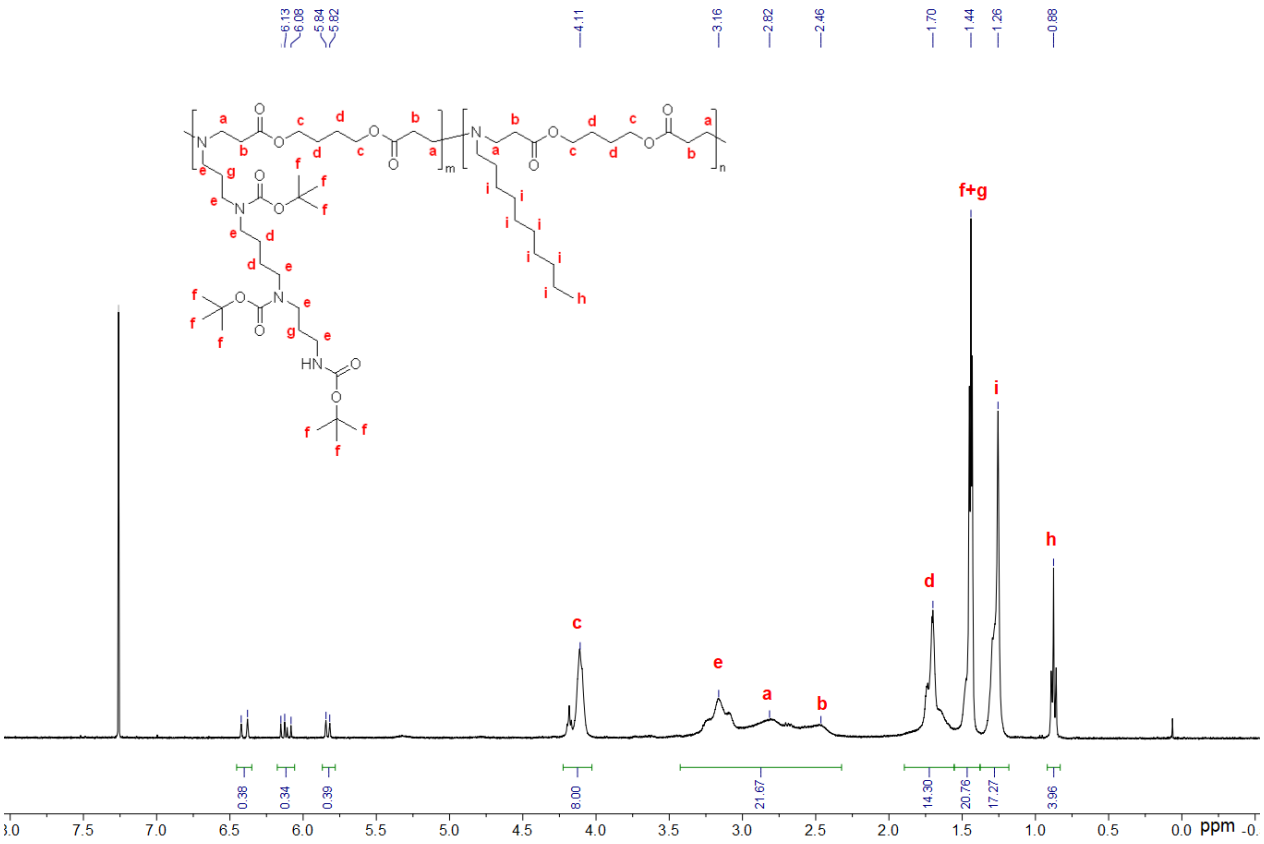
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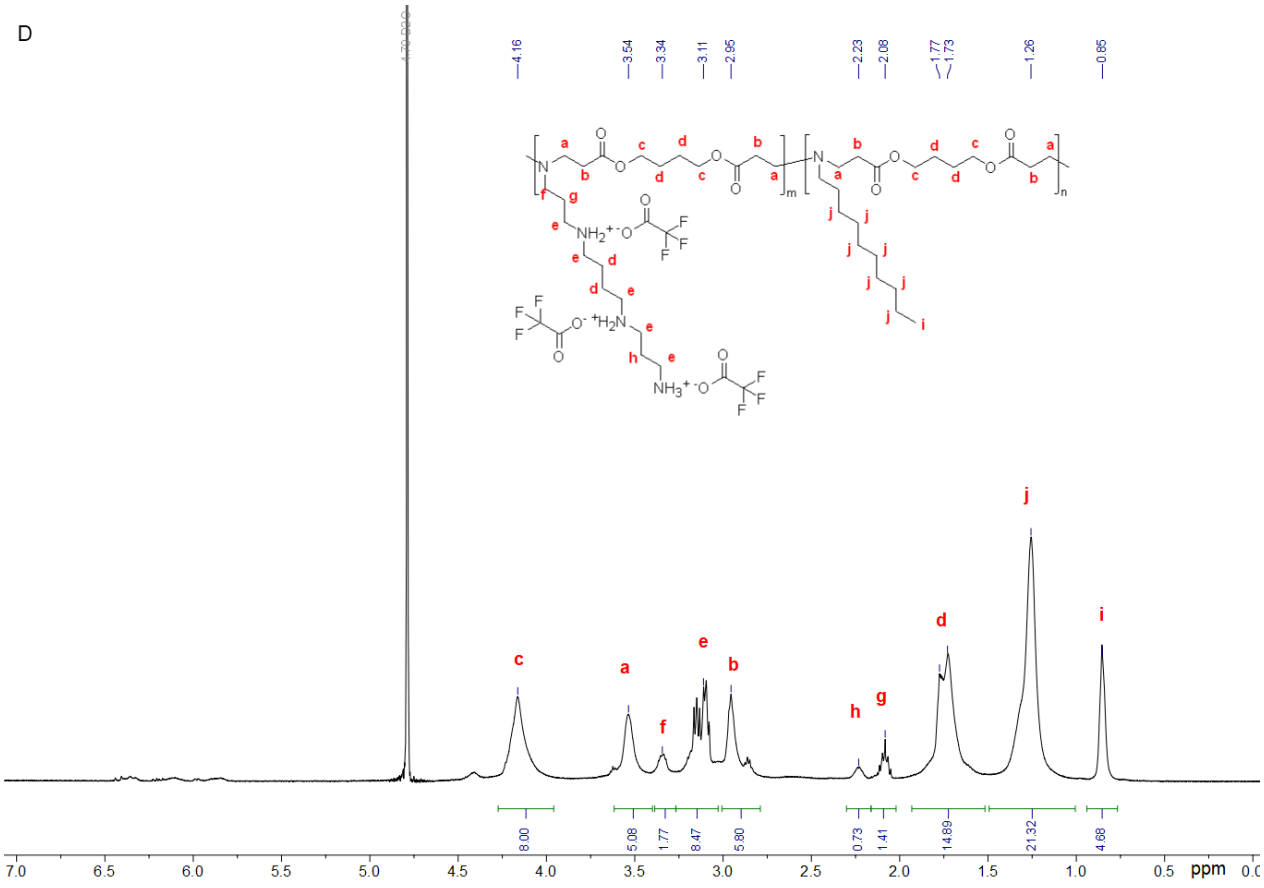

Fig. S1 (A)^{1}H NMR of tri-boc-spermine in $CDCl_3$; (B) ^{1}H NMR of 1,4-butanediol diacrylate in $CDCl_3$ (C) ^{1}H NMR of P(BSpBAE) in $CDCl_3$; (D) ^{1}H NMR of P(SpBAE) in D_2O; The shift of peak C from 4.18 ppm (Fig. S1B) to 4.11 ppm (Fig. S1C) indicated the conjugation of 1,4-butanediol diacrylate with tri-boc-spermine or decylamine.

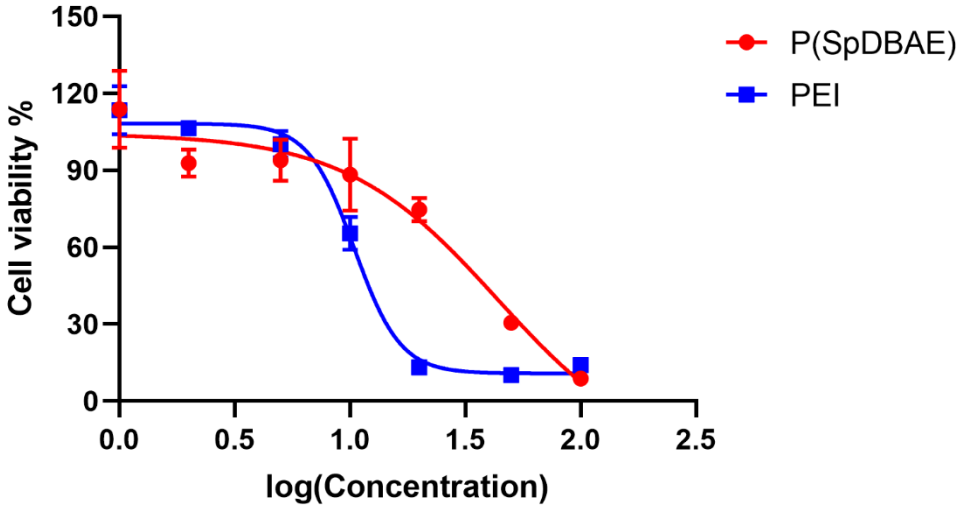

Fig. S2 Cell viability of P(SpDBAE) and PEI determined by MTT assay. IC50: P(SpDBAE) 43.47 µg/mL, PEI 10.45 µg/mL.

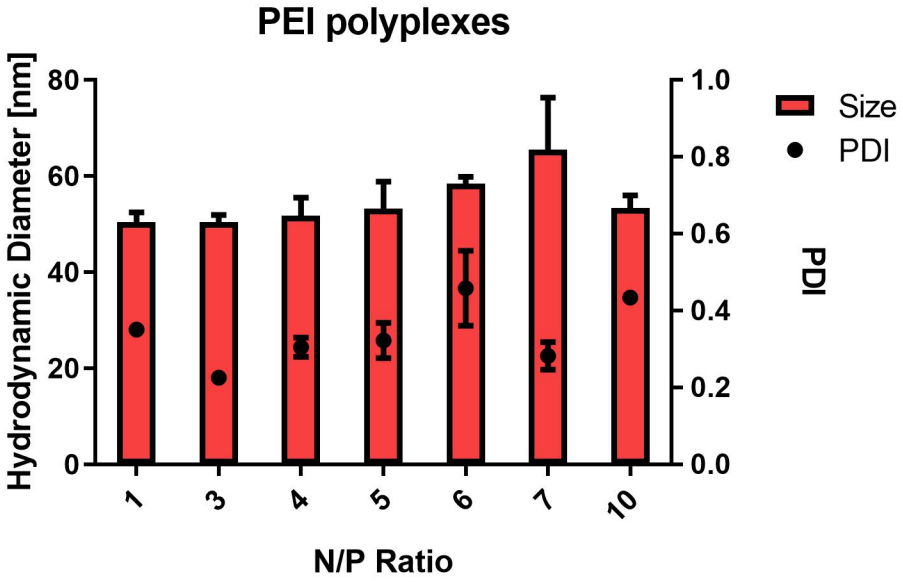

Fig. S3 Hydrodynamic diameter (size) and polydispersity index (PDI) of PEI polyplexes.

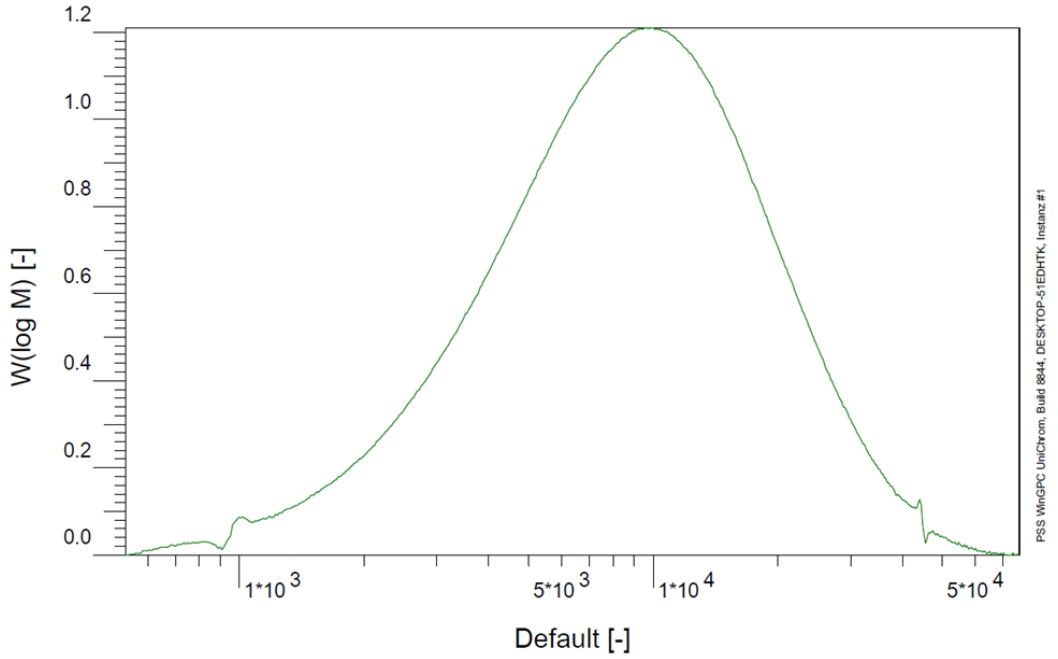

Fig S4. Size exclusion chromatography (SEC) trace of P(BSpDBAE) in chloroform.

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