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Supplementary Material

Peptide-Based Star Polymers as Potential siRNA Carriers

Shu Jie Lam,[A,B] *Adrian Sulistio,*[A] *Katharina Ladewig,*[A] *Edgar H. H. Wong,*[A] *Anton Blencowe ,*[A,C,D] *Greg G. Qiao* [A,D]

[A]Polymer Science Group, Department of Chemical & Biomolecular Engineering, The University of Melbourne, Parkville, VIC 3010, Australia.

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[C]Current Address: Mawson Institute, Division of Information Technology, Engineering and the Environment, University of South Australia, Mawson Lakes, SA 5095, Australia.

[D]Corresponding authors. Email: anton.blencowe@unisa.edu.au; gregghq@unimelb.edu.au

Experimental

Materials.

H-Lys(Z)-OH (>99%, Fluka), generation 2.0 poly(amido amine) (G2 PAMAM) (Dendritech), generation 3.0 poly(amido amine) (G3 PAMAM) (Dendritech), bis(trichloromethyl)carbonate (triphosgene) (99%, Aldrich), MeO-PEG-OH (M_w = 5 kDa) (Aldrich), succinic anhydride (>99%, Aldrich), 4-(dimethylamino) pyridine (DMAP) (99%, Aldrich), pyridine (>99.5%, Scharlau), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI) (>98%, Fluka), lithium

bromide (99%, Aldrich), hydrobromic acid (33% in acetic acid) (Aldrich), pentane (anhyd., >99%, Aldrich), and *N*,*N*-dimethylformamide (DMF) (anhyd., Aldrich) were used as received. Diethyl ether, chloroform, DMF, and methanol were purchased from Chem-Supply and used as received. THF (99%, Lab Scan) was distilled from sodium benzophenone ketal under argon. Milli Q water (18.2 MΩ.cm) was obtained from a Millipore Synergy Water System. Dimetylsulfoxide-d_6 (DMSO-d_6) (99.9%) was purchased from Cambridge Isotope and used as received. MALDI ToF MS matrix 2-[(2E)-3-(4-*tert*-butylphenyl)-2-methylprop-2-enylidene]malonitrile (DCTB) and cationisation agent potassium trifluoroacetate (KTFA) (98%) were purchased from Santa Cruz Biotechnology and Aldrich, respectively, and were used as received. RPMI-1640 medium without L-glutamine (GIBCO Cat. No. 21870), fetal bovine serum (FBS, GIBCO Cat. No. 10099), GlutaMAX™ supplement (100x, GIBCO Cat. No. 35050), antibiotic-antimycotic (100x, GIBCO Cat. No. 15240), MEM non-essential amino acids (100x, GIBCO Cat. No. 11140), Dulbecco's Phosphate Buffered Saline (DPBS, GIBCO 14190), 0.05% trypsin-EDTA (1x, GIBCO Cat. No. 25300), and alamarBlue® cell viability reagent (DAL1025) were purchased from Invitrogen™ and used as received. Certified molecular biology agarose (Cat. No. 1613100), nucleic acid sample loading buffer (Cat. No. 1610767), Tris-acetate-EDTA (TAE) buffer (50x, Cat. No. 1610743), and ethidium bromide solution (Cat. No. 1610433) were purchased from Bio-Rad and used as received. Nuclease-free water (Cat. No. 129114) was purchased from Qiagen and used as received. The double-stranded oligodeoxynucleotide (ODN, concentration: 50 μM) was obtained after ligation of two complementary single strands (strand one 5'→3' TACACCACCAGATGGCAGTGCTCTTCC, reverse strand 5'→3' AGCGGAAGAGCACTGCCATCTGGTGGTGTA) both of which were obtained from Aldrich (custom oligo synthesis).

Instrumentation.

GPC analysis was performed on a Shimadzu liquid chromatography system equipped with a PostNova PN3621 MALS detector (λ = 532 nm), Shimadzu RID-10 refractometer (λ = 633 nm) and Shimadzu SPD-20A UV-Vis detector using three identical Jordi columns (5 μm bead size, Jordi Gel Fluorinated DVB Mixed Bed) in series operating at 70 ^{o}C. The eluent was HPLC grade DMF containing 0.05 M LiBr (1 mL/min). NovaMALS software (PostNova Analytics) was used to determine the molecular weight characteristics using the known refractive index increment (d*n*/d*c*) values for PZLL in DMF (d*n*/dc_{PZLL} = 0.068 mL/g (25°C)). All samples for GPC analysis were prepared at a concentration of 10 mg/mL and were filtered through 0.45 μm nylon filters prior to injection. ^{1}H NMR spectroscopy was performed at room temperature using a Varian Unity400 (400 MHz) spectrometer with the deuterated solvent as reference and a sample concentration of *ca.* 10 mg/mL. DLS measurements were performed on a DynaPro NanoStar instrument (Wyatt Technology) with a He-Ne laser (658 nm) at an angle of 90° and a temperature of 25 ± 0.1°C. Zeta-potential measurements were conducted in MilliQ water using a Zetasizer Nano ZS (Malvern). For both DLS and zeta-potential measurements, initial sample concentrations of 1 mg/mL in MilliQ water were used and serial dilutions were performed until stable spectra were obtained. All sample solutions were filtered using 0.45 μm syringe filters. MALDI ToF MS was performed on a Bruker Autoflex III Mass Spectrometer operating in positive/linear mode. The analyte, matrix (DCTB) and cationisation agent (KTFA) were dissolved in THF at concentrations of 10, 10 and 1 mg/mL, respectively, and then mixed in a ratio of 10:1:1. 0.3 μL of this solution was then spotted onto a ground steel target plate and the solvent was allowed to evaporate prior to analysis. FlexAnalysis (Bruker) was used to analyse the data. Agarose gel electrophoresis was carried out using a Bio-Rad Sub-Cell® GT Agarose

Gel Electrophoresis system. Gels were imaged using a Bio-Rad Molecular Imager® ChemiDoc™ XRS System and captured using Quantity One analysis software. UV-Vis absorbances were determined using a Varian Cary® 50 Bio UV-Visible spectrophotometer equipped with an appropriate microplate reader attachment.

Procedures.

Synthesis of lysine(Z)-NCA (Lys NCA). Dried H-Lys(Z)-OH (1.24 g, 4.43 mmol) was added to anhydrous THF (25 mL) in an oven-dried two-necked round bottomed flask under argon. Triphosgene (580 mg, 1.96 mmol) was dissolved in anhydrous THF (5 mL) and added to the H-Lys(Z)-OH suspension. The mixture was heated at 50 ^{o}C for 30 min with continuous stirring. The clear solution was allowed to cool to room temperature and added to anhydrous pentane (100 mL). The resulting precipitate was isolated via centrifugation and washed with anhydrous pentane (30 mL x 2). The resulting white solid was dried at ambient temperature *in vacuo* to afford Lys NCA, 0.910 g (81%). ^{1}H NMR (400 MHz, d_6-DMSO) δ_H 1.23-1.37 (m, γ-C**H**$_2$, 2H), 1.37-1.45 (m, δ-C**H**$_2$, 2H), 1.60-1.80 (m, β-C**H**$_2$, 2H), 2.94-3.02 (m, ε-C**H**$_2$, 2H), 4.40-4.43 (m, α-C**H**, 1H), 4.98-5.02 (dd, C_6H_5C**H**$_2$-, 2H), 6.90 (s, ring N**H**, 1H), 7.30-7.39 (m, C_6**H**$_5$-, 5H).

General procedure for synthesis of poly(Z-L-lysine)$_{arm}$PAMAM$_{core}$ star polymers. Aqueous stock solutions of the PAMAM dendrimers (PAMAM-$(NH_2)_{16}$ or PAMAM-$(NH_2)_{32}$) were transferred to Schlenk tubes and dried *in vacuo* at room temperature for 4 h, and then at 60 ^{o}C for 1 h. After cooling to room temperature, anhydrous DMF was added (such that the concentrations of the dendrimers are *ca*. 10 mg/mL) with continuous stirring under argon. Lys NCA was dissolved in anhydrous DMF (*ca*. 35 mg/mL) and transferred via syringe into the PAMAM solution under argon. The mixture was stirred at room temperature for 24 h, *n*-butyl

alcohol (1 mL) was added and the stirring was continued for 1 h. The reaction mixture was concentrated *in vacuo* and the resulting star polymer was isolated *via* precipitation into diethyl ether.

*Synthesis of Star Polymer **1***. Lys NCA (1 g, 3.26 mmol) was dissolved in anhydrous DMF (9 mL) and added via syringe to PAMAM-$(NH_2)_{16}$ (33 mg, 10.2 μmol) dissolved in anhydrous DMF (1 mL). After stirring for 24 h, *n*-butyl alcohol (1 mL) was added and the mixture was stirred for a further 1 h. Precipitation of the concentrated polymer solution into diethyl ether (3 x 40 mL), followed by isolation *via* centrifugation and drying (0.1 mbar), afforded $PZLL_{arm}$-PAMAM-$(NH_2)_{16,core}$ star polymer **1** (M_w = 85.6 kDa; PDI = 1.16) as an off-white solid, 770 mg (88%).

*Synthesis of Star Polymer **2***. Lys NCA (1 g, 3.26 mmol) was dissolved in anhydrous DMF (9 mL) and added via syringe to (PAMAM-$(NH_2)_{32}$ (35 mg, 5.1 μmol) dissolved in anhydrous DMF (1 mL). After stirring for 24 h, *n*-butyl alcohol (1 mL) was added and the mixture was stirred for a further 1 h. Precipitation of the concentrated polymer solution into diethyl ether (3 x 40 mL), followed by isolation *via* centrifugation and drying (0.1 mbar), afforded $PZLL_{arm}$-PAMAM-$(NH_2)_{32,core}$ star polymer **2** (M_w = 125 kDa; PDI = 1.23) as an off-white solid, 780 mg (87%).

General Procedure for Determining Monomer Conversion. After 24 h of polymerisation, 100 μL aliquots of the crude star samples for **1** and **2** were taken for ^{1}H NMR analysis before the addition of *n*-butyl alcohol. Resonances of the methine proton (δ_H 4.2 ppm) of the strained Lys NCA and its open polymeric form (δ_H 3.85 ppm) were integrated. The monomer conversion was determined from the ratio of the integrals of these two resonances.

Calculation of the Number of Lysine Repeat Units Per Arm. The average number of lysine repeat units per star arm, n, was calculated using **Equation S1**:

$$n = \frac{M_{\mathrm{w,star}} - M_{\mathrm{w,core}}}{m \times M_{\mathrm{w,lys}}} \qquad (\mathbf{S1})$$

where the star molecular weight ($M_{\mathrm{w,star}}$) was determined by GPC. The molecular weight of the PAMAM dendrimer ($M_{\mathrm{w,core}}$) was either 3256 Da for G2 or 6909 Da for G3. The number of star arms (m) was taken to be either 16 for **1** or 32 for **2**. The molecular weight of the ring-opened lysine ($M_{\mathrm{w,lys}}$) was taken to be 262.31 Da.

Synthesis of MeO-PEG$_{5000}$-COOH. Dried MeO-PEG$_{5000}$-OH (5.00 g, 1.00 mmol) was dissolved in anhydrous 1,4-dioxane (5 mL) under argon. Succinic anhydride (1.00 g, 10 mmol), pyridine (1.58 g, 20.0 mmol) and DMAP (122 mg, 1.00 mmol) were added to the solution at ambient temperature. The reaction mixture was stirred at 45 ^{o}C under nitrogen for 24 h. After cooling to room temperature, the solvent was removed *in vacuo* and the residue was dissolved in chloroform (2 mL). The insoluble solids were filtered off and the filtrate was precipitated into cold diethyl ether (4 ^{o}C). After isolation *via* centrifugation and drying (2 mbar), MeO-PEG$_{5000}$-COOH was obtained a white powder, 2.85 g (56.4%). MALDI-ToF MS: M_w = 5110, PDI = 1.00. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 2.55-2.65 (m, C**H**$_2$CO, C**H**$_2$COOH, 4H), 3.33 (s, C**H**$_3$O, 3H), 3.55-3.74 (m, 113C**H**$_2$O, 452H).

General procedure for synthesis of PEGylated poly(Z-L-lysine)$_{arm}$PAMAM$_{core}$ star polymers. The star polymer (**1** or **2**) was dissolved in anhydrous DMF, and MeO-PEG$_{5000}$-COOH was added and allowed to dissolve under continuous stirring. DMAP and EDCI were then added. After stirring for 24 h, the resulting mixture was precipitated into diethyl ether, centrifuged and dried *in vacuo* to afford the PEGylated star polymers.

Synthesis of PEGylated star polymer $\mathbf{1}_{PEG}$**.** Starting from star polymer **1** in anhydrous DMF (38 mg/mL), MeO-PEG$_{5000}$-COOH (808 mg, 158 μmol), DMAP (10.6 mg, 87 μmol) and EDCI (167 mg, 915 μmol) were added. Precipitation of the polymer solution into diethyl ether (40 mL) followed by isolation *via* centrifugation and drying (0.1 mbar) afforded $\mathbf{1}_{\mathbf{PEG}}$, 550 mg (74%).

Synthesis of PEGylated star polymer $\mathbf{2}_{PEG}$**.** Starting from star polymer **2** in anhydrous DMF (20 mg/mL), MeO-PEG$_{5000}$-COOH (2.2 g, 427 μmol), DMAP (28.7 mg, 235 μmol) and EDCI (450 mg, 2.35 mmol) were added. Precipitation of the polymer solution into diethyl ether (40 mL) followed by isolation *via* centrifugation and drying (0.1 mbar) afforded $\mathbf{2}_{\mathbf{PEG}}$, 653 mg (71%).

Calculation of the Extent of PEGylation. The average extent of PEGylation (% PEG) for each star was calculated using **Equation S2**:

$$\%\ \mathrm{PEG} = \frac{M_{\mathrm{w,star-PEG}} - M_{\mathrm{w,star}}}{M_{\mathrm{w,PEG}}} \qquad (\mathbf{S2})$$

where the PEGylated star molecular weight ($M_{\mathrm{w,star\text{-}PEG}}$) and the precursor star molecular weight ($M_{\mathrm{w,star}}$) were determined by GPC relative to polystyrene standards. The average molecular weight of the carboxylated PEG ($M_{\mathrm{w,PEG}}$) was taken as 5110 Da.

General procedure for deprotection of star polymers 1, **2**, $\mathbf{1}_{\mathbf{PEG}}$, and $\mathbf{2}_{\mathbf{PEG}}$**.** The star polymer (either **1**, **2**, $\mathbf{1}_{\mathbf{PEG}}$, or $\mathbf{2}_{\mathbf{PEG}}$) was dissolved in TFA (200 mg/mL) and 33% HBr in acetic acid was then added (20 mL/g star). After 24 h stirring at room temperature, the mixture was precipitated into diethyl ether (10 times the volume of the reaction). The precipitate was isolated *via* centrifugation, redissolved in methanol (2 mL) and precipitated into THF (30 mL). After repeating this step thrice, the residue was dried *in vacuo* (2 mbar) to obtain the deprotected star polymer (either $\mathbf{1}_{\mathbf{d}}$, $\mathbf{2}_{\mathbf{d}}$, $\mathbf{1}_{\mathbf{PEG,d}}$, or $\mathbf{2}_{\mathbf{PEG,d}}$).

ODN retardation experiments. Double-stranded ODNs and the star polymers $\mathbf{1_d}$, $\mathbf{2_d}$, $\mathbf{1_{PEG,d}}$, and $\mathbf{2_{PEG,d}}$ were mixed in various nitrogen/phosphorus (N/P) ratios ranging from 1:1 to 50:1. Deionised water was added to afford a final volume of 10 μL, such that the final ODN concentration was 0.1 g/L. After incubation at 37 ^{o}C for 10 min with light agitation (350 min^{-1}), an appropriate amount of nucleic acid sample loading buffer was added to each sample. Agarose gel electrophoresis was then carried out using 3% agarose gels (TAE buffer, 1% ethidium bromide) and gels were imaged as described above.

Cell culture. Human embryonic kidney cells (HEK293T) were cultivated in 'complete' RPMI-1640 medium (supplemented with 5% FBS, 1x GlutaMAX^{TM}, 1x antibiotic-antimycotic, and 1x MEM non-essential amino acids) in a humidified atmosphere containing 5% CO_2 at 37 ^{o}C. Cells were seeded in a T175 flask (*ca*. 3 x 10^6 cells/ml) and passaged twice a week prior to the performance of the subsequent cell viability studies.

Cell viability study. Cytotoxicity of the polymers was assessed using Invitrogen's alamarBlue® cell viability reagent following the manufacturer's instructions. Briefly, a 'mixing plate' was prepared in which 1.56 x 10^{-6} M of polymer stock solutions were added to the rightmost column and serially diluted across a 96 well plate, resulting in 11 successive 1:2 dilutions across the plate. In another 96 well plate, 'the experiment plate', 80 μL of cell suspension was seeded in each well (*ca.* 10,000 cells/well) except for the 'medium blanks' in which 80 μL of medium was added instead. 20 μL of the corresponding well on the 'mixing plate' was then added to the cell suspension on the 'experiment plate' which was incubated in humidified atmosphere containing 5% CO_2 at 37 ^{o}C. After 48 h, 10 μL of alamarBlue® cell viability reagent was added to each well (except for three wells containing medium only). After 3-3½ h of incubation under the same growth conditions, the absorbance at 570 and 600 nm of each well was measured using a Varian

Cary 50 Bio UV-Visible spectrophotometer. The absorbances of each well were corrected against the medium-only wells without alamarBlue® reagent and the R_o correction factor for each plate was calculated as follows: R_o = Corrected λ_{570} of alamarBlue® in media/Corrected λ_{600} of alamarBlue® in media. Subsequently, the amount of reduced alamarBlue® (AR_{570}) was calculated for each well as follows: AR_{570} = Corrected λ_{570} – (Corrected λ_{600} x R_o). AR_{570} for each well was then expressed as a percentage of growth control. Note that all experiments were conducted in triplicates on three different occasions and error bars shown represent the standard error of the mean of independent experiments.

Structure of a Generation 2 PAMAM Dendrimer

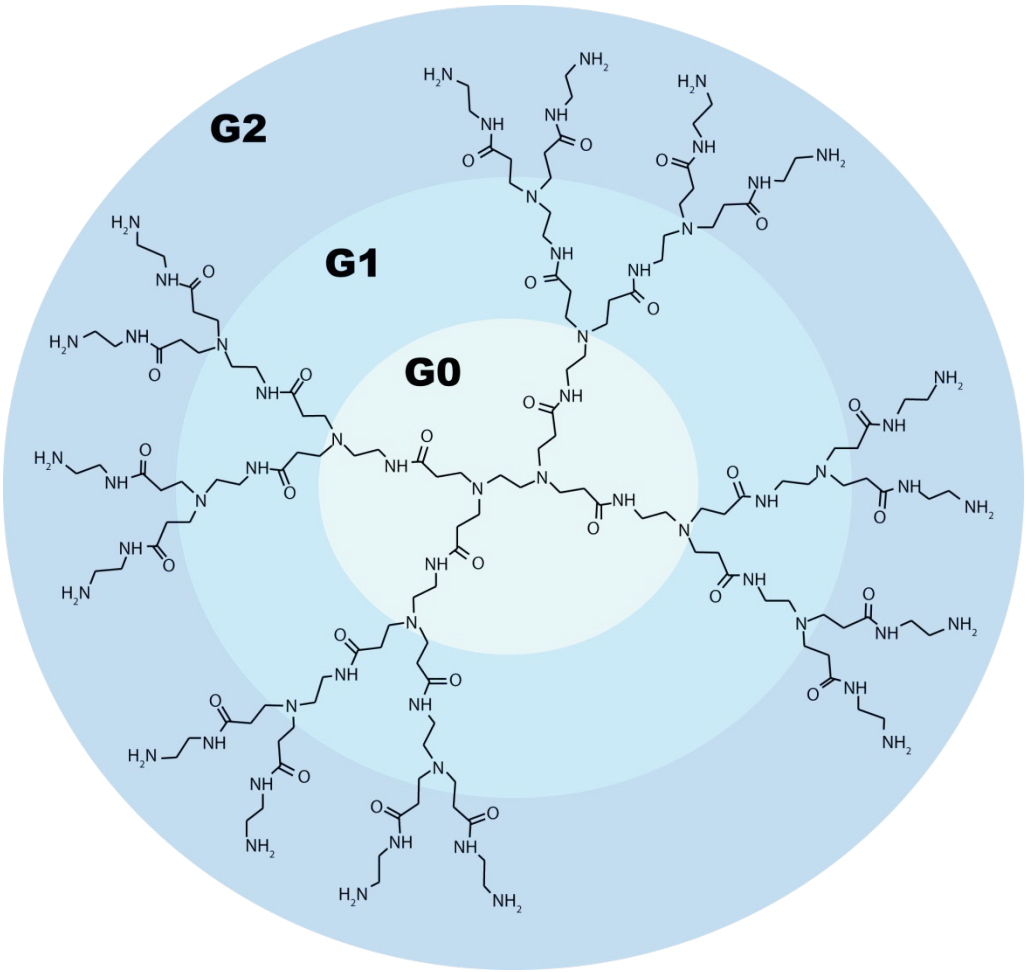

Fig. S1. Structure of a generation 2 PAMAM dendrimer (G0 = generation 0; G1 = generation 1; G2 = generation 2)

Characterisation of Star Polymers 1 and 2

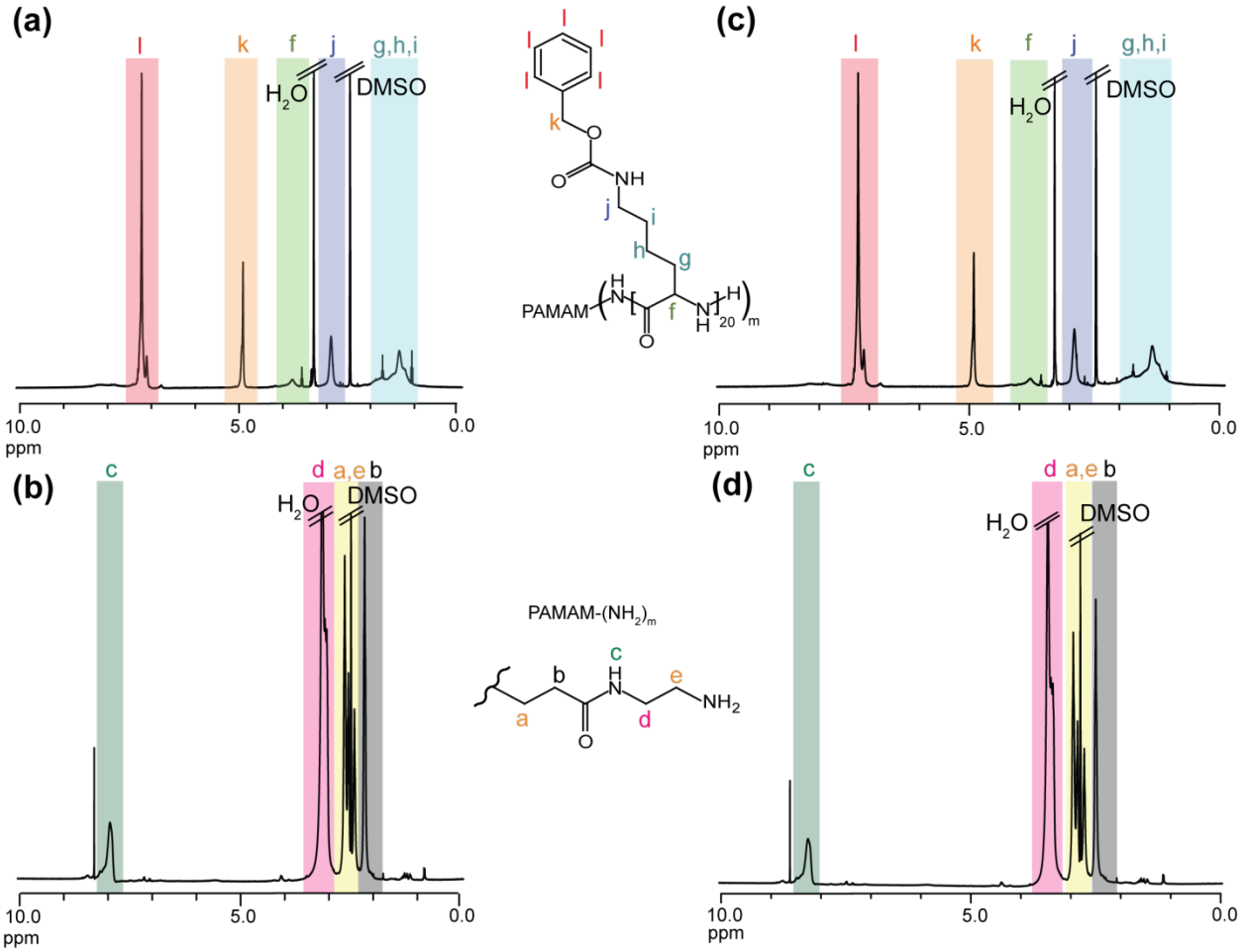

Fig. S2. ^{1}H NMR spectra (d_6-DMSO) of (a) 16-arm star polymer **1**, (b) G2 PAMAM dendrimer, (c) 32-arm star polymer **2**, and (d) G3 PAMAM dendrimer.

Characterisation of MeO-PEG$_{5000}$-COOH

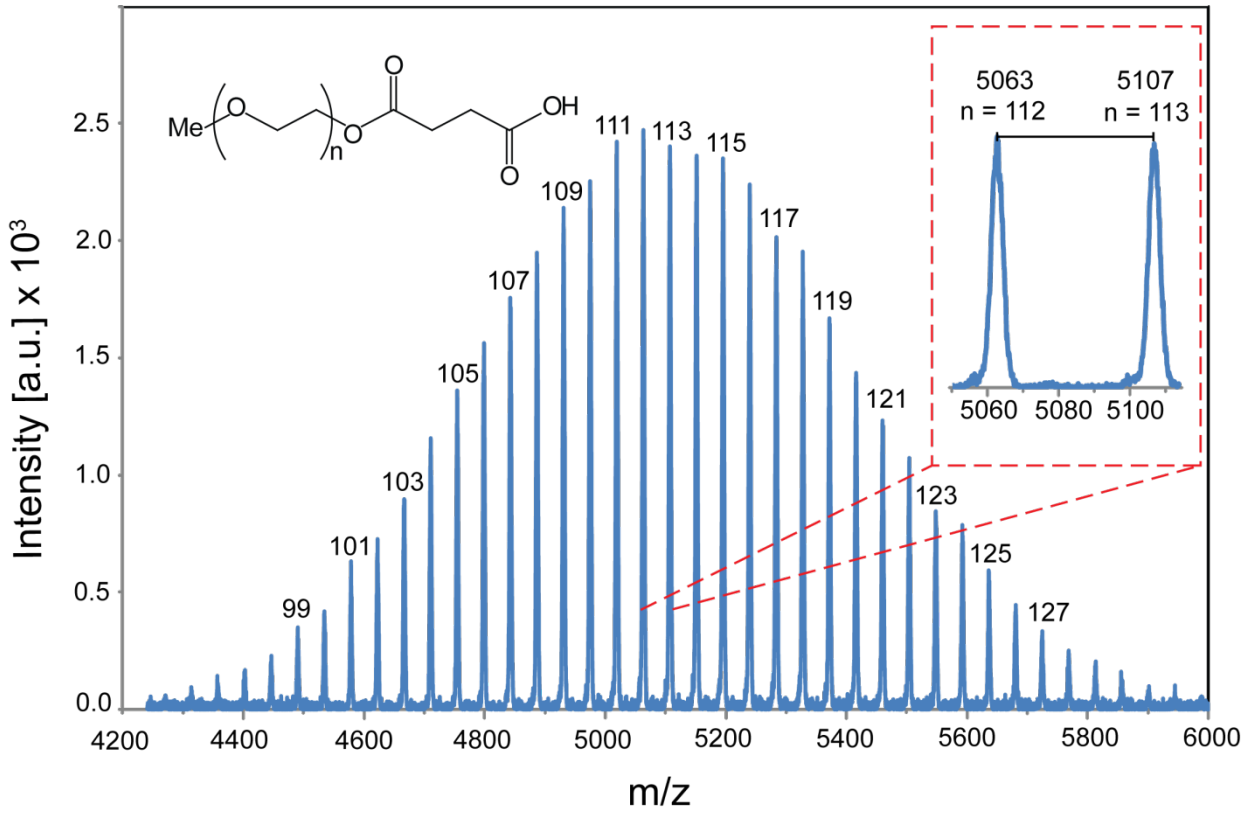

Fig. S3. MALDI ToF mass spectrum of MeO-PEG$_{5000}$-COOH recorded in linear/positive mode using DCTB and KTFA as the matrix and cationisation agent, respectively. The numbers on the spectrum indicate the number of PEG repeat units (n, 44.03 m/z). Inset shows an expanded section of the spectrum with peak m/z values and the m/z difference.

Processed GPC DRI Chromatograms for Star Polymers 1 and 2

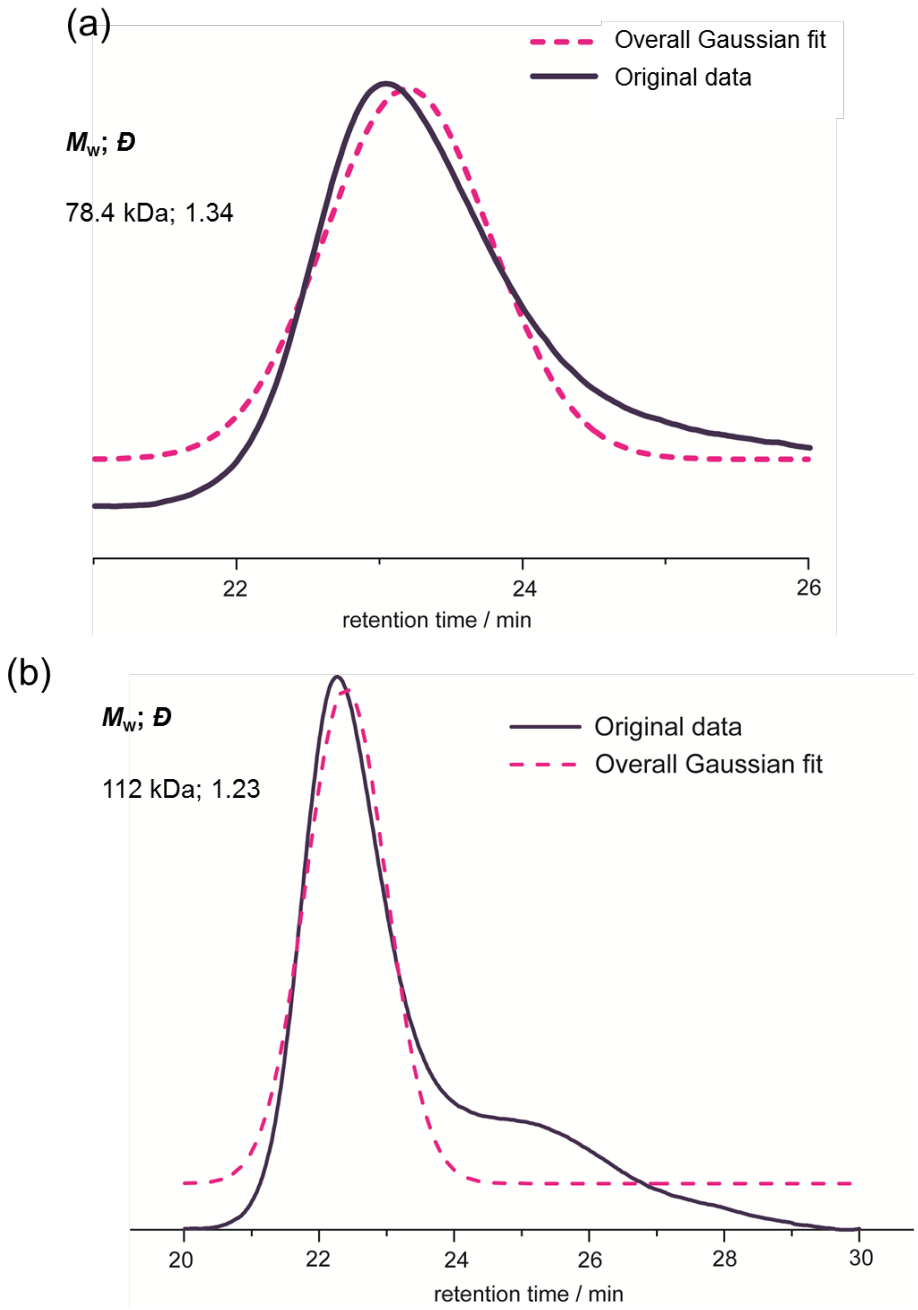

Fig. S4 Deconvoluted GPC DRI chromatograms of unPEGylated star polymers (a) **1** and (b) **2**. Deconvolution was performed using OriginPro by employing an overall Gaussian fit. The weight-average molecular weight (M_w) and dispersity ($Đ$) values refer to the deconvoluted unPEGylated stars **1** and **2** and were calculated based on polystyrene standards.

Processed GPC DRI Chromatograms for Star Polymers $\mathbf{1}_{PEG}$ and $\mathbf{2}_{PEG}$

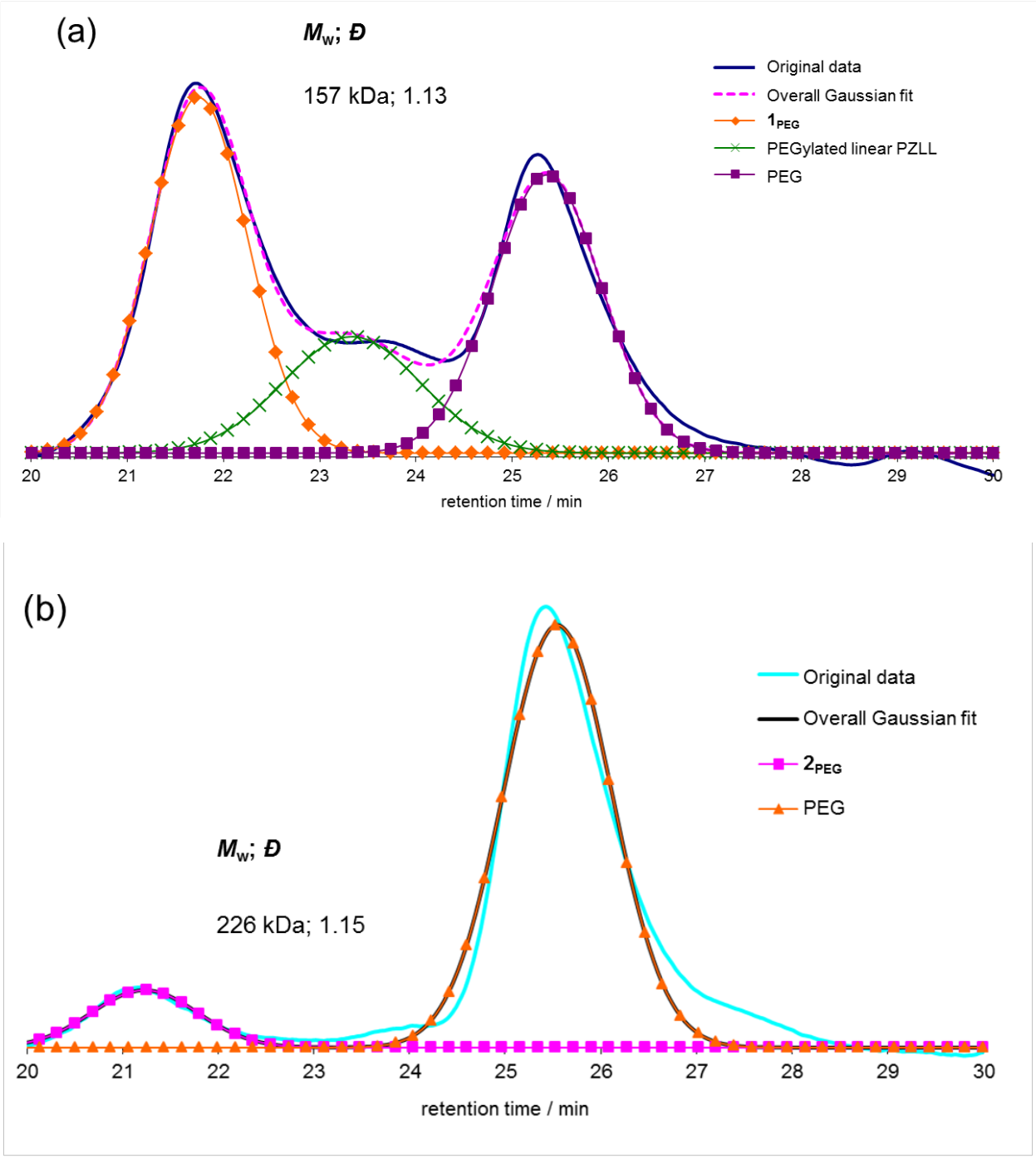

Fig. S5 Deconvoluted GPC DRI chromatograms of PEGylated star polymers (a) $\mathbf{1}_{\mathbf{PEG}}$ and (b) $\mathbf{2}_{\mathbf{PEG}}$. Deconvolution was performed using OriginPro by employing an overall Gaussian fit. The weight-average molecular weight (M_w) and dispersity ($Đ$) values refer to the deconvoluted PEGylated stars $\mathbf{1}_{\mathbf{PEG}}$ and $\mathbf{2}_{\mathbf{PEG}}$ and were calculated based on polystyrene standards.

Characterisation of UnPEGylated Star Polymers Before and After Deprotection

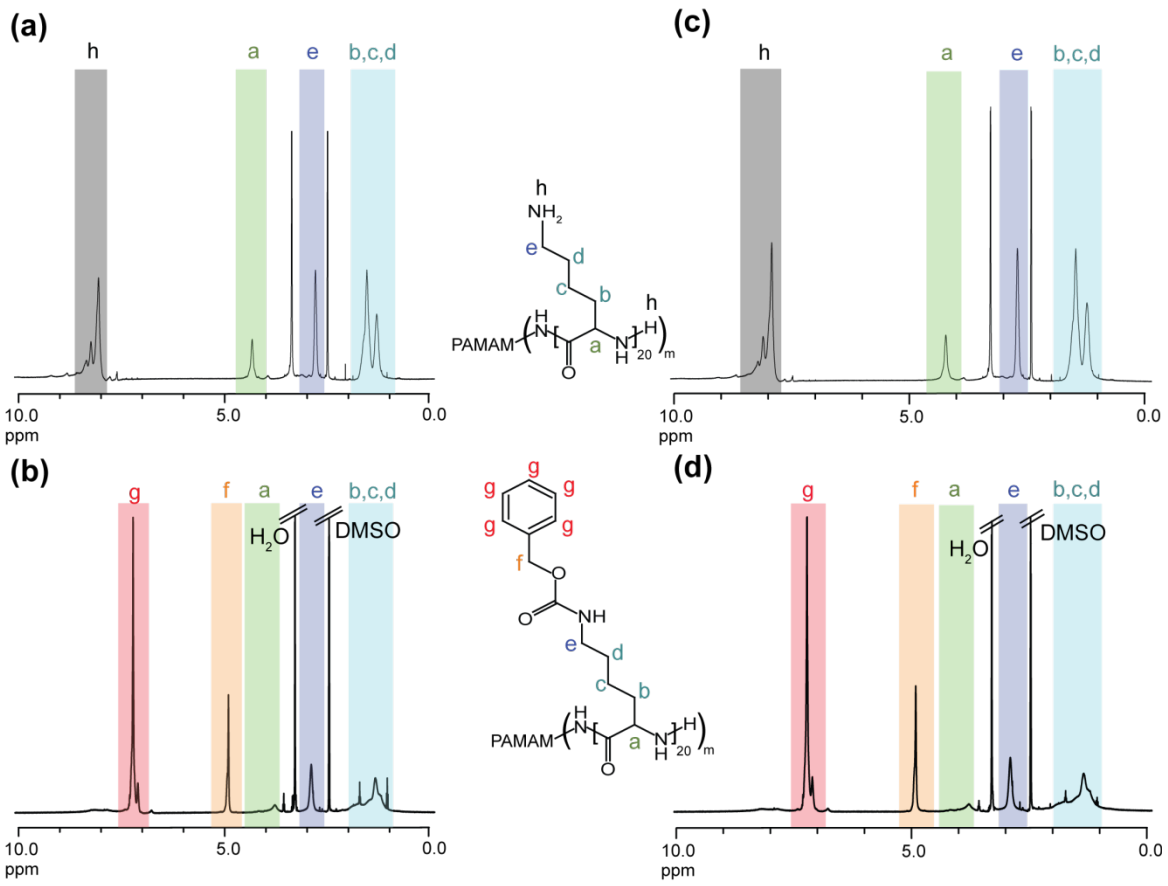

Fig. S6 ^{1}H NMR spectra (d_6-DMSO) of the 16-arm (a) deprotected star polymer $\mathbf{1_d}$ and (b) Cbz-protected star polymer **1**, and the 32-arm (c) deprotected star polymer $\mathbf{2_d}$ and (d) Cbz-protected star polymer **2**.

Characterisation of PEGylated Star Polymers Before and After Deprotection

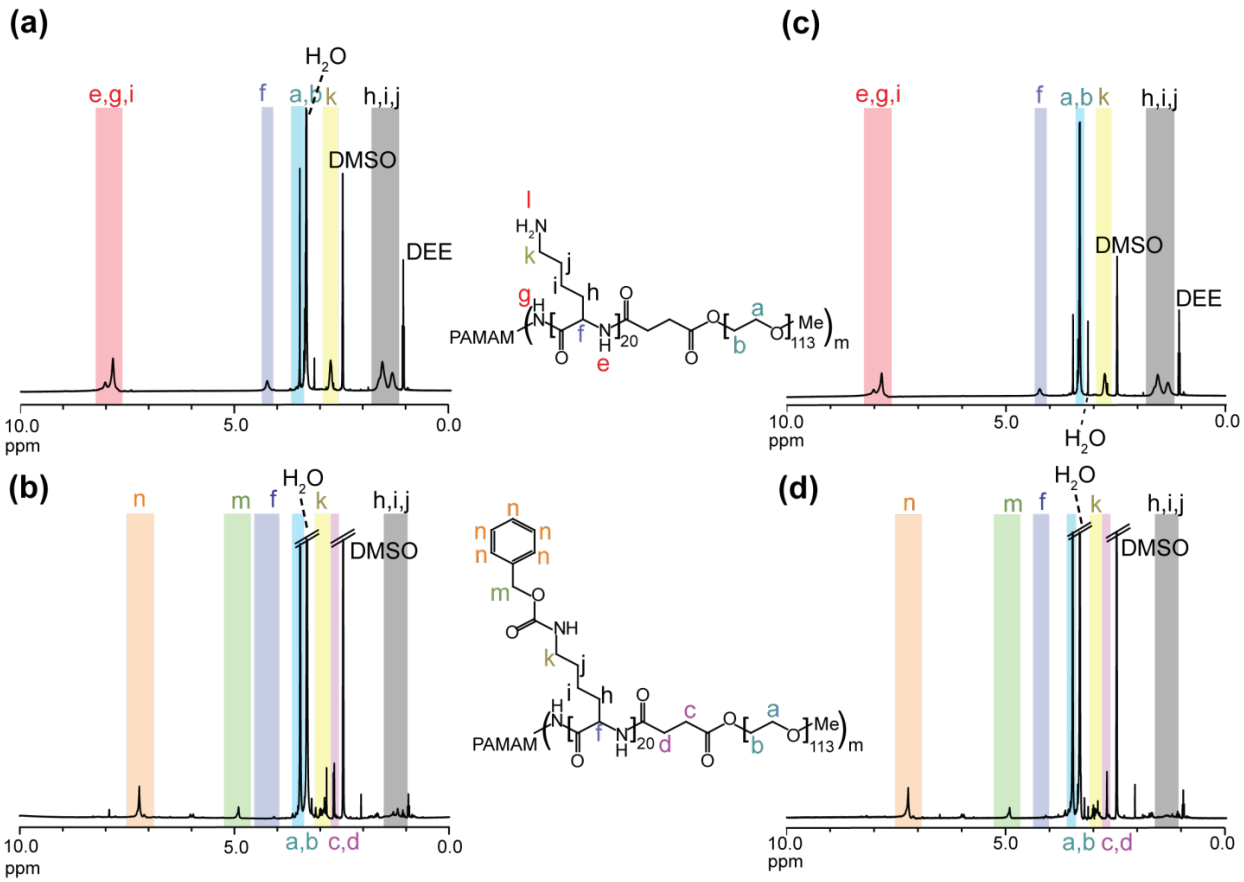

Fig. S7. ^{1}H NMR spectra (d_6-DMSO) of 16-arm (a) deprotected PEGylated star polymer $\mathbf{1}_{\mathbf{PEG,d}}$ and (b) Cbz-protected PEGylated star polymer $\mathbf{1}_{\mathbf{PEG}}$, and the 32-arm (c) deprotected PEGylated star polymer $\mathbf{2}_{\mathbf{PEG,d}}$ and (d) Cbz-protected PEGylated star polymer $\mathbf{2}_{\mathbf{PEG}}$.

Zeta Potential Distribution

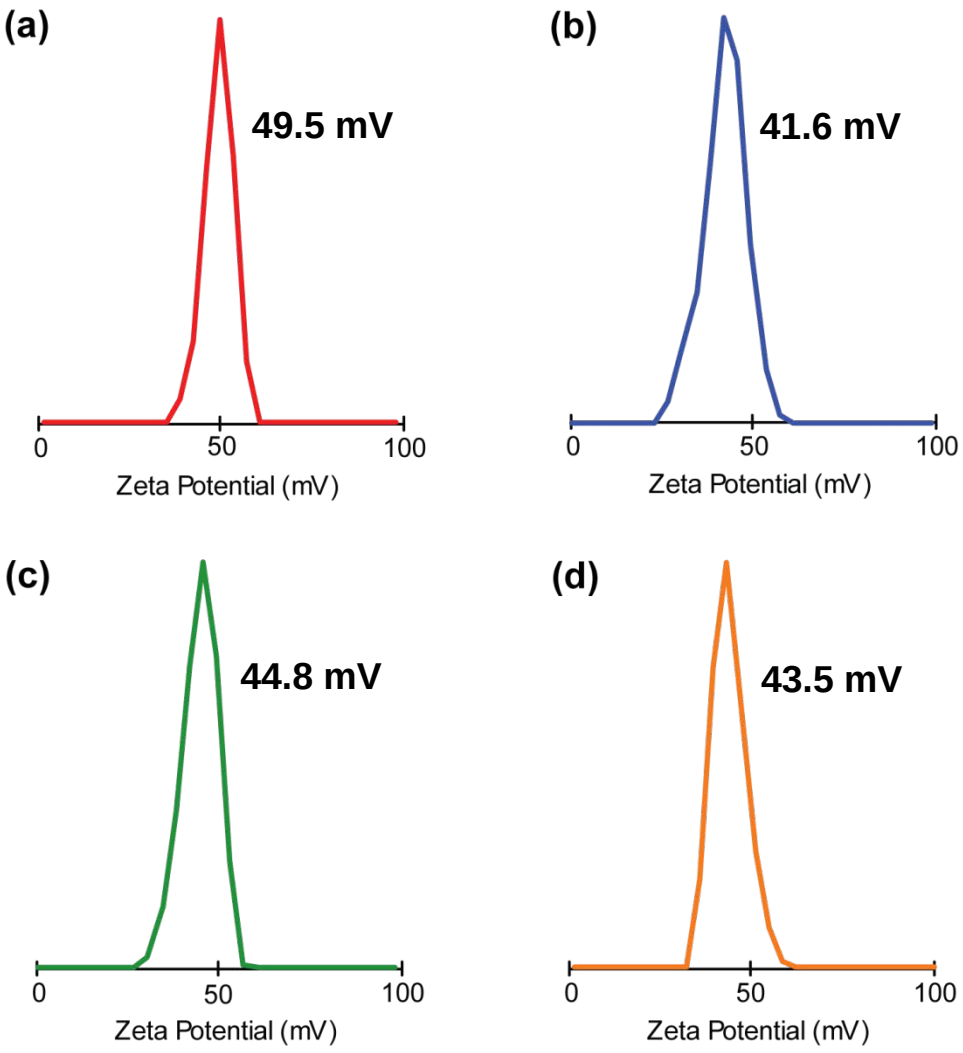

Fig. S8. Zeta potential distribution of (a) 16-arm star polymer $\mathbf{1}_{\mathbf{d}}$, (b) 32-arm star polymer $\mathbf{2}_{\mathbf{d}}$, (c) 16-arm PEGylated star polymer $\mathbf{1}_{\mathbf{PEG,d}}$, and (d) 32-arm PEGylated star polymer $\mathbf{2}_{\mathbf{PEG,d}}$.