Supporting Information

Zwitterionic polymers with high serum tolerance for intracellular protein delivery

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Methods

Materials

G4 and G5 polyamidoamine (PAMAM) dendrimers were obtained from Dendritech (Midland, MI). 25-Kda branched polyethyleneimine (bPEI) and bafilomycin A1 (BafA1) were got from Sigma Aldrich (St. Louis, MO, USA). β-Galactosidase (β-Gal) and 4-diethylaminophenyl isothiocyanate (DAITC) were procured from J&K Scientific (Shanghai, China). 2-Formylbenzenesulfonic acid was acquired from Alfa Aesar (Thermo Fisher Scientific, USA). Dimethyl sulfoxide (DMSO) was obtained from Sangon Biotech, Co. Ltd. (Shanghai, China). 1-methoxy-5-methylphenazinium methyl sulfate (mPMS) was purchased from Bidepharm. (Shanghai, China). Cytochalasin D (CytoD), Chlorpromazine (CPZ), and Methyl-β-cyclodextrin (MβCD) were sourced from MedChemExpress (Monmouth Junction, NJ, USA). 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8), Lysotracker Red, Hoechst 33342, and β-galactosidase staining kit were acquired from Beyotime (Shanghai, China). 4-Fluorophenyl isothiocyanate, phenyl isothiocyanate, 2-naphthaldehyde, sodium borohydride cyanoborohydride, p-aminobenzoic acid, acetic acid, and hydrochloric acid were purchased from Shanghai Adamas Reagent.

(Shanghai, China). FITC, RBITC, genistein, and sodium acetate were got from Macklin (Shanghai, China). TransExcellent (TransEx) was purchased from Cenji Biotech (Shanghai, China). NIH3T3 and HeLa cells were obtained from American Type Culture Collection (ATCC). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO (Gaithersburg, MD).

Polymer synthesis

Hydrophobic ligands bearing an isothiocyanate group (4-fluorophenyl isothiocyanate, phenyl isothiocyanate, and 4-diethylaminophenyl isothiocyanate) were mixed with 50 mg polymers (G4, G5 PAMAM dendrimers or bPEI) and then dissolved in 4 mL anhydrous DMSO according to the feed molar ratios shown in Tables S1 and S3. The mixture was stirred at room temperature for 24 h. The products were dialyzed twice in DMSO (MWCO 3500, biosharp) for subsequent reactions, or protonated by hydrochloric acid and purified by intensive dialysis in DI water. The purified products were freeze-dried and characterized by ^{1}H NMR in D_2O (Bruker Avance 500, Germany).

The polymers were further modified with 2-formylbenzenesulfonic acid or aldehyde-benzoic acid according to the following procedures. 2-formylbenzenesulfonic acid or aldehyde-benzoic acid was dissolved in 4 mL anhydrous DMSO and then mixed with 50 mg polymers according to the feed ratios shown in Tables S1 and S3. The reaction mixtures were stirred overnight at room temperature after the addition of 30 μL acetic acid. Then cyanoborohydride sodium was added into the above solutions at a cyanoborohydride/aldehyde molar ratio of 1.5:1, and the mixtures were stirred for additional 24 h at room temperature. The resulting products were dialyzed twice in DMSO (MWCO 3500), protonated by hydrochloric acid, purified through intensive dialysis in DI water. The purified products were freeze-dried and characterized by ^{1}H NMR in D_2O.

Characterization of protein complexes

The synthesized polymers and EGFP of different concentrations (Table S2) were mixed and incubated at room temperature for 30 min. The zeta potential and size of the complexes were characterized via Malvern Zetasizer Nano ZS90 in H_2O. The formed complexes incubated in

DMEM or DMEM supplemented with 10% FBS were observed through a Zeiss LSM880 (German) confocal laser scanning microscope (CLSM).

Cell culture and cytosolic protein delivery

NIH3T3 cells and HeLa cells expressing Rab5-RFP were cultured in DMEM medium (containing 10% FBS, 1% penicillin sulfate and streptomycin) at 37 °C, 5% CO_2. Cells were cultured in 48-well plates or 35-mm glass-bottom confocal dishes at an 80% density prior to protein delivery. The working solutions of GFS and proteins for cell treatment were fixed at 2 mg/ml and 1 mg/ml, respectively. Protein complexes were prepared with 15 μg GFS and 10 μg EGFP or 10 μg GFS and 5 μg β-gal. The complexes were incubated with the cells in different serum-containing media at 37°C for 6 hours. After incubation, the medium was removed, and the cells were washed twice with PBS. The cells were then treated with a PBS solution containing 0.05% Trypan blue for 5 minutes and rinsed with PBS. The fluorescence intensity of the cells was measured using a flow cytometer (BD FACS Verse, USA). Additionally, the treated cells were observed by confocal laser scanning microscopy (CLSM) or fluorescence microscopy (Leica, DM IL, Germany).

Endocytosis and intracellular trafficking

Generally, NIH3T3 cells were incubated with different inhibitors such as genistein (18.9 μg/mL), CPZ (7 μg/mL), MβCD (10 mg/mL), and cytochalasin D (1 μg/mL) in DMEM medium at 37°C for 2 h before protein delivery. Untreated cells were tested as a control. Subsequently, cells were incubated with protein complexes for 4 h and then imaged by fluorescence microscopy or analyzed by flow cytometry. The time-dependent protein delivery experiments were investigated using CLSM. Typically, cells were incubated with protein complexes for 1-6 h, and then stained with Hoechst 33342 and LysoTracker Red for 20 min, washed with PBS, and imaged using CLSM.

Cell viability assay

Cytotoxicity of the polymers and EGFP complexes was evaluated by the WST-8 assay. NIH3T3 cells were cultured in 96-well plates at a density of 10^4 per well overnight. Different concentrations of polymers and complexes were added to a 96-well plate and incubated with the cells for 48 hours. After incubation, the prepared CCK-8 solution was added to each well and incubated for 2 hours at 37°C. The absorbance of the solutions at 450 nm was then measured using a microplate reader (Multiskan Sky, Thermo Fisher Scientific, USA).

Protein absorption assay

Fluorescence resonance energy transfer (FRET) assay was used to evaluate the protein adsorption behavior of each polymer complexes. Generally, 20 μg of polymer and 10 μg of ECFP were incubated in 30 μL of ultrapure water for 5 min first. Subsequently, 50 μL of phenol-red-free MEM medium was added, and incubated for 1 h. Afterward, 20 μg of EYFP was introduced into the above solutions and further incubated for 2 h at room temperature. Then the solutions were diluted with ultrapure water to a total volume of 1 mL, and the diluted solutions were transferred into a transparent quartz dish, and the fluorescence emission spectra were recorded using a fluorescence spectrophotometer (F-320, China) with an excitation wavelength of 405 nm.

SDS-PAGE assay was also used to evaluate the protein adsorption of polymer/EGFP complexes. Generally, 60 μg of polymers were mixed with 40 μg of EGFP in a 1.5 mL centrifuge tube. Ultrapure water was added to the complex solutions to reach a total volume of 200 μL. The mixture was incubated for 5 minutes, followed by the addition of 500 μL PBS (pH 7.4) and stabilization for 1 hour. Subsequently, FBS was added to the centrifuge tubes to achieve a 50% FBS concentration, and the mixtures were left at room temperature for either 6 or 24 hours. Afterward, the solutions were centrifuged at 15,000 rpm for 5 minutes to remove the supernatants. The resulting precipitates were washed three times with PBS and then mixed with protein loading buffers. The protein mixtures were then heated in a metal bath at 100°C for 5 minutes. Finally, the samples were separated by electrophoresis on a 10% SDS-PAGE gel and observed after staining with Coomassie Brilliant Blue.

Statistical analysis

Statistical analysis was conducted using a one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test for groups larger than two. For comparisons between two groups, the Student's t-test was employed. Values of $p < 0.05$ were considered as significance.

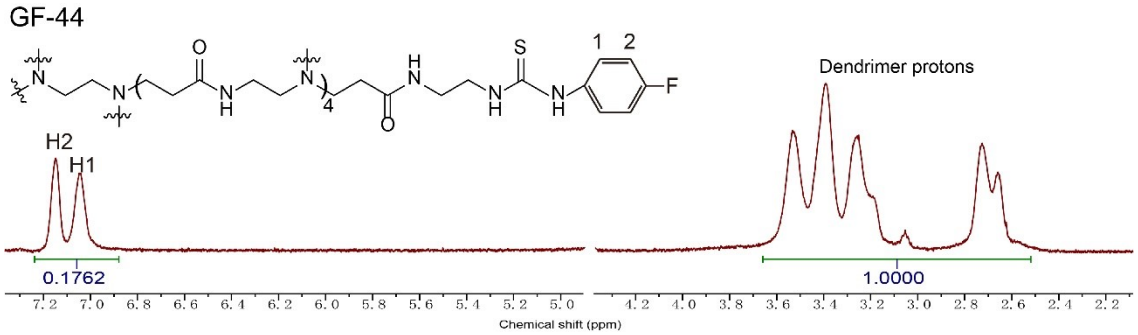

Figure S1. ^{1}H NMR spectrum of GF-44 in D_2O.

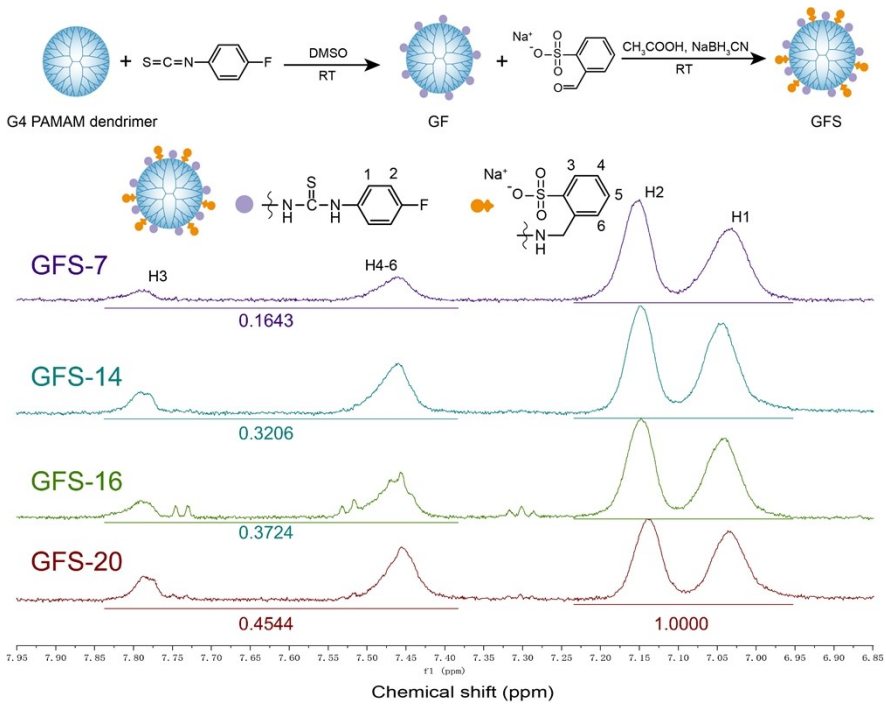

Figure S2. Synthetic route and ^{1}H NMR of GFS series polymers in D_2O.

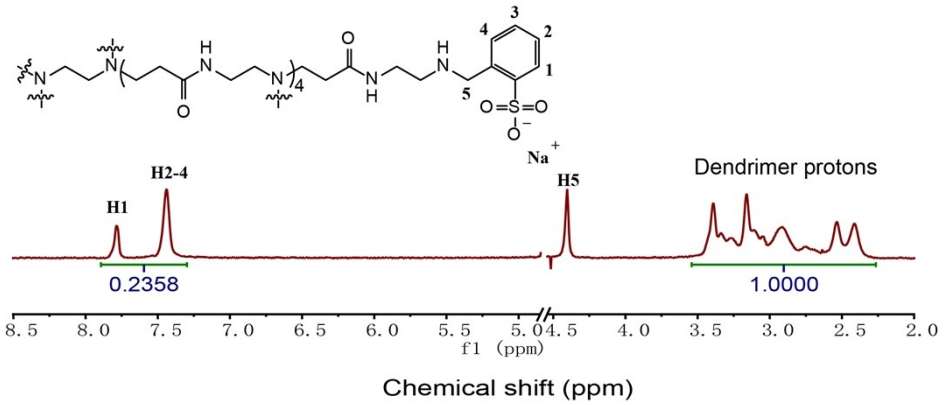

Figure S3. ^{1}H NMR of GS58 polymers in D_2O.

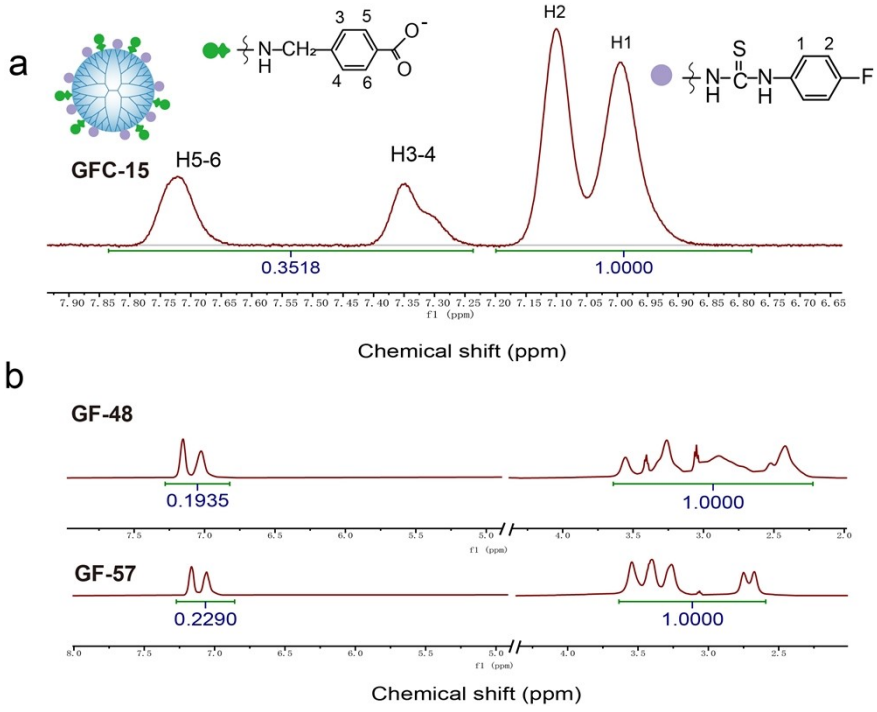

Figure S4. ^{1}H NMR spectra of (a) GFC-15, (b) GF-48 and GF-57 in D_2O.

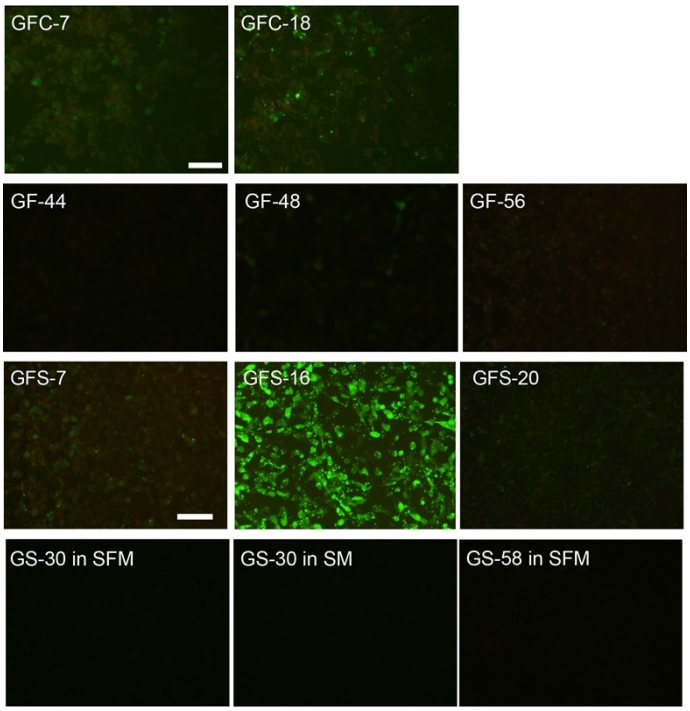

Figure S5. a) EGFP delivery by different polymers on NIH3T3 cells. The amount of EGFP was 10 μg per well. The doses of GFC-7, GFC-15 and GFC-18 were 15 μg per well. Protein complexes were incubated in 48-well plates with NIH3T3 cells for 6 h in medium containing 10% FBS. Scale bar, 100 μm.

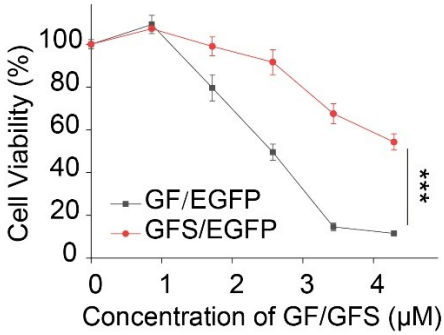

Figure S6. Cytotoxicity of GF/EGFP and GFS/EGFP complexes on NIH3T3 cells (n=5, ***p<0.001).

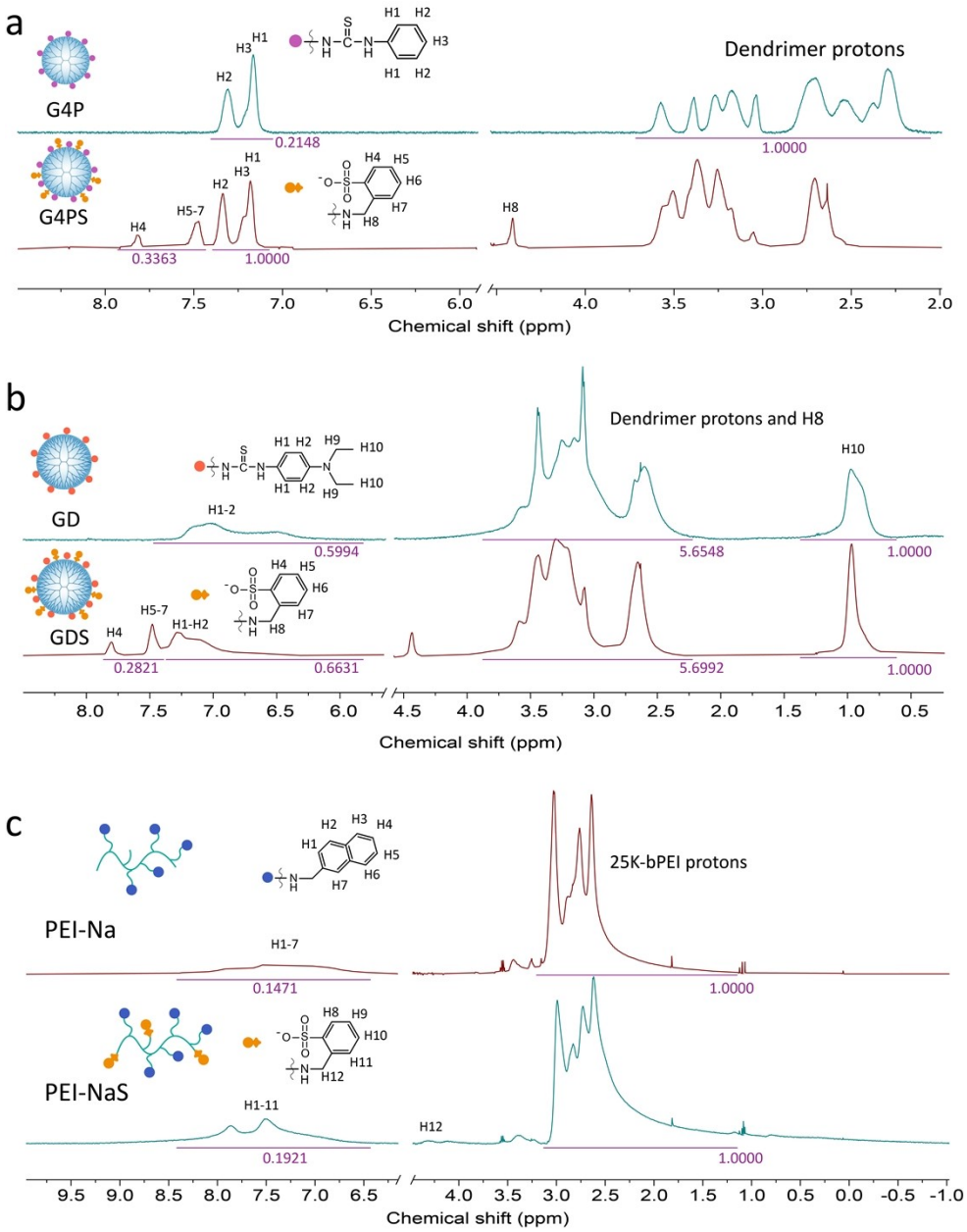

Figure S7. ^{1}H NMR spectra of the synthesized polymers (a) GP and GPS; (b) GD and GDS; (c) PEI-Na and PEI-NaS in D_2O.

Table S1. Feeding molar ratios of chemicals to G4 PAMAM dendrimer during the synthesis of GF and GFS series polymers.

Name	Conjugated 4-fluorophyl on each G4	Feeding molar ratio of benzenesulphonate to G4	Conjugated benzenesulphonate on each G4	Number of residual primary amines on G4
GF	44	0	0	20
GFS-7	44	7	7	13
GFS-14	44	14	14	6
GFS-16	44	16	16	4
GFC-15	44	20	15	5
GF-50	48	0	0	16
GF-57	57	0	0	7
GFS-20	44	20	20	0
GS-58	0	80	58	6

Table S2. Size and particle distribution index of GFS/EGFP nanoparticles prepared at different concentrations.

Concentration of carrier（mg/mL）	Concentration of EGFP（mg/mL）	Incubation time（h）	Hydrated diameter（nm）	PDI
2	1	0.5	857	0.22
1	1	0.5	600	0.22
0.5	1	0.5	686	0.20
0.1	1	0.5	643	0.17
2	0.5	0.5	409	0.19
2	0.2	0.5	486	0.17
2	0.1	0.5	687	0.15
0.1	0.2	0.5	254	0.04
0.1	0.1	0.5	142	0.07
0.1	0.2	24	244	0.08
0.1	0.1	24	144	0.09

Table S3. Feeding molar ratios of chemicals to cationic polymers

Name	Conjugated hydrophobic groups on polymer	Feeding molar ratio of benzenesulphonate to G4	Conjugated benzenesulphonate on each G4	Number of residual primary amines on G4
G4P	43	0	0	21
G4PS	43	20	18	7
GD	68	0	0	60
GDS	68	40	28	32
PEI-Na	49	0	0	--
PEI-NaS	49	30	26	--