Supporting Information

Zwitterion and Oligo(ethylene glycol) Synergy Minimizes Nonspecific Binding of Compact Quantum Dots

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1 Supporting Methods

Materials. The following materials and reagents were purchased from Sigma-Aldrich: 1-octadecene (ODE, 90% tech.), 1-octanethiol (OT, 98.5%), oleylamine (OLA, 70%), cadmium acetate dihydrate ($Cd(Ac)_2 \cdot 2H_2O$, 98%), sulfur powder (S, 99.98%), selenium dioxide (SeO_2, ≥99.9%), 1,2-hexadecanediol (HDD, 97%), behenic acid (BAc, 99%), *N*-hydroxysuccinimide (NHS), acryloyl chloride, triethylamine (TEA), 2-cyano-2-propyl benzodithioate (CTA), 2,2'-azobis(2-methylpropionitrile) (AIBN), trifluoroacetic acid (TFA), histamine, *N*-Boc-ethylenediamine, 3-aminopropionic acid (β-alanine), tetramethylammonium hydroxide solution (TMAH, 25 wt % in methanol), *N*,*N*,*N*,*N*-tetramethylethylenediamine (TMEDA), *N*,*N*'-dicyclohexylcarbodiimide (DCC), poly(acrylic acid) (PAA), anhydrous dimethylformamide (DMF), DMSO, BSA, and LPS. Oleic acid (OAc, 90%), anhydrous cadmium chloride ($CdCl_2$, 99.99%), and zinc acetate ($Zn(Ac)_2$, 99.98%) were purchased from Alfa Aesar. N^{α}-Boc-L-2,3-diaminopropionic acid (Boc-Dap-OH), N^{α}-Boc-L-2,4-diaminobutyric acid (Boc-Dab-OH), N^{α}-Boc-L-lysine (Boc-Lys-OH) were purchased from Chem-Impex. Monoamine-, monohydroxy-triethylene glycol (NH_2-EG_3-OH) was purchased from BroadPharm. Agarose, ammonium persulfate (APS) and acrylamide/*N*,*N*'-methylenebisacrylamide (19:1, 40% mix solution in water) and paraformaldehyde (PFA, 32% in water) were from Bio-Rad Laboratories Inc. Solvents including NMF, acetone, hexane, diethyl ether, chloroform and methanol were purchased from Acros Organics or Fisher Scientific. PBS was from Corning. His-tag PA (catalog No. 6500) was purchased from BioVision. αEGFR-Ab (mouse IgG, catalog no. 555996) was purchased from BD Bioscience. Control antibody 1 (mouse IgG, catalog no. 02-7102) was purchased from Invitrogen. αiNOS-Ab (rabbit IgG, catalog no. PA5-16524), Hoechst, and Triton X-100 were purchased from Thermo Fisher. Alexa Fluor 594 AffiniPure Donkey Anti-Rabbit IgG (H+L) (catalog no. 711-585-152) was purchased from Jackson ImmunoResearch Laboratory. Control antibody 2 (rabbit IgG, catalog no. ab172730) was purchased from Abcam.

Synthesis of *N*-Acryloxysuccinimide (NAS). The synthesis scheme for NAS is depicted in **Figure S9**. In a 300 mL flask, NHS (5.75 g, 50 mmol) and TEA (7 mL, 50 mmol) were dissolved in dichloromethane (DCM, 100 mL). Acryloyl chloride (4.6 mL, 55 mmol) was slowly added to the solution on ice and the reaction was allowed to proceed for 5 h. The suspension was filtered and washed twice with cold deionized water (30 mL × 2) and cold saturated brine (30 mL). The organic layer was dried over anhydrous Na_2SO_4 and the filtrate was condensed to 20 mL by rotary evaporation under vacuum.[1] ^{1}H NMR ($CDCl_3$, δ, ppm, 500 MHz): 6.64 (d, 1H, CH_2=C*H*-), 6.13-

6.29 (m, 2H, C*H*$_2$=CH-), 2.78 (br, 4H, -C*H*$_2$C*H*$_2$-); ^{13}C NMR ($CDCl_3$, δ, ppm, 400 MHz): 169.5 (CH*C*=OO), 161.5 (CH_2*C*=ON), 136.9 (*C*H_2=CH-), 123.7 (CH_2=*C*H-), 25.8 (-*C*H_2-).

Synthesis of Poly(NAS) (PNAS). The synthesis scheme for NAS is depicted in **Figure S9**. In a 10 mL Schlenk tube, NAS (1.0 g, 6.0 mmol), CTA (44 mg, 0.2 mmol) and AIBN (3.2 mg, 0.02 mmol) were dissolved in anhydrous DMF (2 mL). The resulting solution was a purple-red color. The Schleck tube was filled with nitrogen in a Dewar filled with liquid nitrogen and then evacuated with an oil pump. This purging and evacuation process was repeated three times. The solution was then purged with nitrogen and the reaction was allowed to proceed at 70 °C. After 8 h, the polymer solution was cooled to room temperature and the product was precipitated with ether (50 mL) and recovered by centrifugation. The polymer was washed again with ether and dried under vacuum (0.85 g, yield 85%). The number average molecular weight was 13.0 kDa (degree of polymerization = 75) with polydispersity index of 1.06.

Synthesis of Amphiphilic Polymer. The amphipol polymer was synthesized following methods previously described in the literature.[2] Briefly, PAA (5 g, 69.39 mmol carboxylic acid) was dissolved in 1-methyl-2-pyrrolidone (NMP, 150 mL) at 50 °C before dropwise addition of octylamine (3.587 g, 27.75 mmol) in NMP (15 mL). The solution was stirred for 1 h and bubbled with nitrogen for 20 min. During bubbling, a solution of DCC (6.872 g, 33.31 mmol) in NMP (10 mL) was added dropwise. The reaction was then bubbled with nitrogen for 10 min and the reaction was allowed to proceed at 50 °C for 24 h. The solution was then cooled in an ice-bath, filtered to remove the dicyclohexylurea byproduct, and diluted in 1.5 L sodium hydroxide (NaOH) aqueous solution (pH 11). The polymer was precipitated by adjusting the pH of the solution to ~3.5 with 5 M HCl and collected by filtration. The polymer was dissolved again in basic solution. This dissolution/precipitation procedure was repeated three times. The purified polymer was then lyophilized, dissolved in chloroform, filtered, and dried under vacuum.

Synthesis of Cadmium Behenate ($Cd(BAc)_2$). $Cd(BAc)_2$ was prepared following literature methods[3] with some modifications. BAc (10 mmol) was dissolved in methanol (200 mL) with the addition of TMAH solution (~3 mL). The mixture was sonicated and stirred for 15 min to fully dissolve BAc. The solution was centrifuged and the colorless supernatant was transferred to a beaker and stirred at 60 °C until the odor of TMAH disappeared. In a separate beaker, $CdCl_2$ (5 mmol) was dissolved in a mixture of methanol and water (20 mL, 4:1 methanol/water). Then the $CdCl_2$ solution was added dropwise to the BAc solution under vigorous stirring. The reaction mixture was stirred for an addition 1 h, generating a white precipitate that was collected by vacuum

filtration and washed several times with methanol on a filter funnel, dried for several hours, and finally dried under vacuum overnight. The product was collected as a white powder.

Synthesis of CdSe Cores. CdSe cores were synthesized following literature methods.[3] $Cd(BAc)_2$ (0.2 mmol), SeO_2 (0.2 mmol), HDD (0.2 mmol), and ODE (6 mL) were transferred to a 25-mL round bottom flask. The mixture was degassed at ~100 °C for 1 h before the temperature was ramped to 240 °C at a rate of ~20 °C/min under nitrogen atmosphere. The mixture was allowed to react for 1 h at 240 °C. The reaction mixture was then cooled to ~100 °C and OAc (1 mL) was added into the reaction flask. The mixture was cooled to room temperature and transferred to a 50 mL centrifuge tube. A mixture of methanol (5 mL) and acetone (30 mL) was added and the mixture was centrifuged at 7000 *g* for 5 min at room temperature to precipitate the CdSe cores. The pellet was dispersed in hexane (5 mL), and again precipitated with methanol and acetone. The resulting purified CdSe cores were dispersed in hexane, yielding a QD dispersion with first exciton absorption band at 538 nm and fluorescence emission band at 549 nm.

Shell Growth on CdSe Cores. A shell of CdS and CdZnS was grown epitaxially on the CdSe cores following literature methods with some modifications.[2] Cadmium and zinc precursor solutions (0.1 M) were prepared by dissolving cadmium acetate dihydrate or zinc acetate, respectively, in oleylamine under nitrogen. A sulfur precursor solution (0.1 M) was prepared by dissolving sulfur powder in ODE under nitrogen. Purified CdSe cores (~300 nmol) dispersed in hexane (5 mL), ODE (4 mL), and OLA (2 mL) were then transferred to a 50-mL round bottom flask, and the mixture was evacuated at 45 °C until no hexane remained. Then the temperature was increased to 120 °C under nitrogen, at which point layer-by-layer shell growth was initiated in increments of 0.8 monolayers (ML). Precursors solution volumes for each increment were calculated based on the volume of the shell growth per monolayer and the number of CdSe cores present in the reaction solution. The first shell growth step was for sulfur, added dropwise in 3 to 5 min, followed by 15 min of reaction. Next, the same volume of cadmium precursor solution was added in a similar manner and allowed to react for 15 min to complete the first 0.8 ML of CdS shell growth. The reaction temperature was increased stepwise by 10 °C between each precursor additions until reaching a maximum of 190 °C. The shell was finally composed of 2.4 ML of CdS, 0.8 ML of $Cd_{0.8}Zn_{0.2}S$, and 1.5 ML layers of ZnS. After each cycle of growth, an aliquot (50 µL) was withdrawn and diluted 30-fold in hexane to monitor the absorption and emission spectra. After completing the reaction, the mixture was cooled to room temperature and the resulting QDs with peak fluorescence emission at 608 nm were purified by precipitation with methanol and acetone, and then redispersion in hexane.

Amphipol Coating of Quantum Dots. QDs were encapsulated in amphipol-based micelles following methods previously described in the literature.[2] In a 20 mL vial, purified QDs in chloroform (1 μM) were vigorously stirred during dropwise addition of a chloroform solution of amphipol (20 mg/mL) to achieve a 2000-fold molar excess of amphipol relative to QDs. The vial was sealed and with a rubber stopper punctured with a needle and placed in a vacuum desiccator for slow evaporation of chloroform with house vacuum without stirring. After the residue was completely dry, a NaOH solution (10 mM) was added at a volume of 3 mL per nmol of QD to homogeneously disperse the QDs. These QDs were purified by gel permeation chromatography (Superose 6 Increase column with PBS mobile phase) to eliminate empty micelles, and further purified by filtration using an Amicon filter (100 kDa MWCO) five times with phosphate buffer as the diluent.

PEGylation of Amphipol-Coated Quantum Dots. The carboxyl-functionalized amphipol-QDs were PEGylated by mixing the QD dispersion (1 μM, 200 μL in phosphate buffer) with monoamine-terminated methoxy-PEG (2 μmol, 2 kDa, Rapp Polymere) before addition of a freshly prepared solution of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) in DMSO (10 mg/mL, 27.6 μL). The molar QD/PEG/DMTMM ratio was $1{:}10^4{:}5\times10^3$. The mixture was stirred at room temperature for 2 h before addition of a second DMTMM solution to ensure dense PEGylation of the QD. Finally, Tris buffer (1 M, 1 mL) was added to quench the reaction, and the mixture was incubated for 30 min before the QDs were purified by filtration five times using an Amicon filter (100 kDa MWCO) using phosphate buffer as the diluent.

Monodentate Thiol Coating of Quantum Dots. Purified QDs in hexane were transferred to NMF with TMAH as described in Methods. A dispersion of purified QDs in NMF (1 nmol, 270 μL) was added to a solution of mPEG-SH (5 μmol, 2 kDa, Biochempeg) in DMSO (1.5 mL) in a vial sealed with a rubber stopper. The mixture was repeatedly evacuated and purged with nitrogen before heating at 60 °C for 3 h under nitrogen. The reaction mixture was then cooled and the QDs were precipitated with mixed solvent of diethyl ether and chloroform (5:1 v/v). The QDs were then dispersed homogeneously in phosphate buffer (10 mM, 4 mL) and purified by filtration five times using an Amicon filter (100 kDa MWCO) with phosphate buffer as the diluent.

Photophysical Stability Analysis. For photostability measurements, QDs (10 nM in phosphate buffer) were continuously excited at a wavelength of 488 nm while the photoluminescence intensity at 608 nm was recorded using a spectrofluorometer. For pH-dependent photoluminescence measurements, a QD stock (1 μM in phosphate buffer) was diluted in Britton-

Robinson buffer[4] with pH between 4–8, prepared from a mixture of acetic acid (40 mM), boric acid (40 mM), and phosphoric acid (40 mM), titrated to the desired pH using 0.2 M NaOH. Photoluminescence spectra were then collected for each sample with excitation at a wavelength of 365 nm. For glutathione-dependent and culture medium-dependent photoluminescence measurements, a QD stock (1 μM in phosphate buffer) was diluted in complete growth medium (DMEM with 10% FBS and 1% P/S) or phosphate buffer with or without 10 mM glutathione, and photoluminescence spectra were recorded with excitation at a wavelength of 365 nm.

Quantum Yield (QY) Measurements. Relative QY was measured in comparison with fluorescein in 0.1 M NaOH solution as a reference standard with QY of 92%, following methods described in previous reports.[2,5]

Cytotoxicity Assay. Cytotoxicity of polymer-coated QDs was measured as metabolic rate using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent in HeLa cells. Cells were seeded at 10,000 per well in a 96 well plate and treated in triplicate with complete medium（DMEM with 10% FBS and 1% P/S) containing QDs with one of four different coatings, or complete medium containing doxorubicin hydrochloride (Cayman Chemical Company) as a control. After 24 h, the medium was removed and cells were washed with PBS. An MTT stock (5 mg/mL) diluted in medium to 0.5 mg/mL was added to each well and the plate was incubated at 37 °C for 4 h. The medium was then removed, cells were washed with PBS, and DMSO (200 μL) was added to dissolve formazan crystals for 15 min at 37°C in the dark. Fluorescence in each well was measured at 570 nm using a microplate reader.

Evaluation of QD-PA Conjugate Stability by FCS. QDs coated with $p(C_4ZW)_1(OEG)_1$ (1 μM in PBS) were mixed with His-tag PA at a 4:1 molar PA/QD ratio. The reaction was allowed to proceed overnight. The solution was then diluted to a QD concentration of 100 nM, 10 nM, or 1 nM in PBS. After different times (0, 1, 4, 8, 24 h), the QDs with concentration of 1 μM, 100 nM, and 10 nM were rapidly diluted to 1 nM in PBS and FCS time-course data were collected. For QDs initially diluted to 1 nM, no additional dilution was performed prior to FCS data acquisition. Autocorrelation functions were fit to a single component model of Brownian motion, and diffusion coefficients were used to calculate hydrodynamic diameters using the Stokes-Einstein equation.

QD-PA Nonspecific Binding Evaluation by Flow Cytometry. QDs (1 μM in PBS) were mixed with His-tag PA at molar PA/QD ratios of 1, 2, 4, and 8. The reactions were allowed to proceed overnight. Nonspecific binding was evaluated by flow cytometry. HeLa were fixed with PFA (4%,

20 min, room temperature), permeabilized with Triton X-100 (0.1% in PBS, 10 min, room temperature), and blocked with BSA solution (1% in PBS) for 1 h. QD-PA solutions were added to a final concentration of 40 nM. After incubation at room temperature for 2 h, the cells were washed with PBS to remove the unbound QD-PA, and were then resuspended in PBS. The cells were filtered through FALCON Corning 5 mL 12 X 75 mm tubes with 35 μm cell-strainer caps and analyzed by flow cytometry.

Instrumentation. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian U400, VXR500, or U500 NMR spectrometer. Gel permeation chromatography (GPC) for polymer characterization was performed using an instrument equipped with a Model 1200 isocratic pump (Agilent Technology) in series with a 717 Autosampler (Waters) and size exclusion columns (102 Å, 103 Å, 104 Å, 105 Å, 106 Å Phenogel columns, 5 μm, 300 × 7.8 mm, Phenomenex) which were maintained at a temperature of 60 °C. A DAWN HELEOS (Wyatt Technology) multiangle laser light scattering (MALLS) instrument operating at a wavelength of 658 nm and an Optilab rEX refractive index detector (Wyatt Technology) operating at a wavelength of 658 nm were used as detectors. The mobile phase consisted of DMF with 0.1M LiBr at a flow rate of 1 mL/min. Samples were filtered through a 0.45 μm PTFE filter before analysis. Absolute molecular weights of polymers were determined using ASTRA 6.1.1.17 software (Wyatt Technology) and calculated from dn/dc values assuming 100% mass recovery. Fluorescence spectra were acquired from hexane-dispersed samples using a NanoLog Horiba Jobin Yvon with Fluo Essence V3.5 software (HORIBA Scientific). Absorption spectra were collected from hexane-dispersed samples using a Cary series UV-Vis-NIR spectrophotometer with Cary WinUV Scan Application Version 6.00 1551 software (Agilent Technologies). Transmission Electron Microscopy (TEM) images were acquired using a JEOL 2010 LaB6 high resolution microscope. For QDs in organic solvents, samples were prepared by placing a drop of dilute QD solution in hexane on an ultrathin carbon film TEM grid (Ted Pella catalog no. 01824) and then wicking the solution off with a tissue. Fluorescence assays in 96-well plates were analyzed using a BioTek Synergy HT Fluorescence Microplate Reader.

2 Supporting Figures and Tables

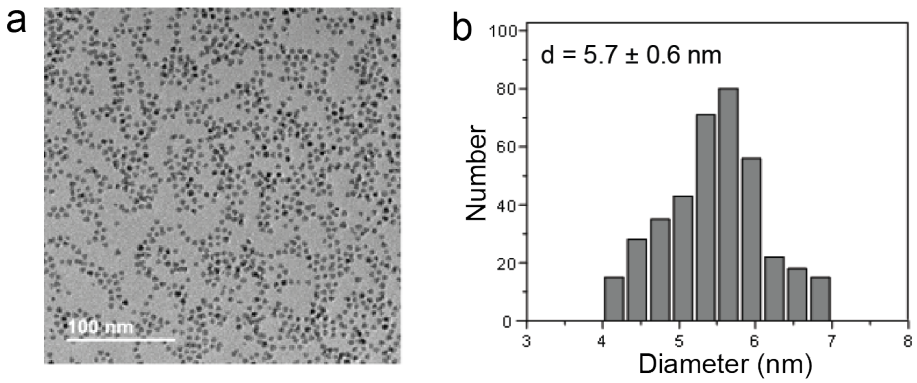

Figure S1. Electron microscopy characterization of QDs used in this work, showing **(a)** a representative micrograph and **(b)** size histogram. QDs were deposited from a hexane dispersion.

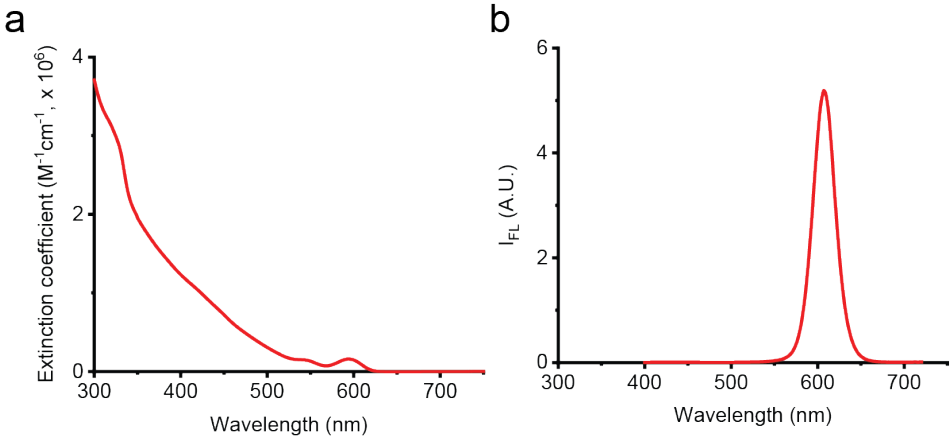

Figure S2. Optical characterization of QDs used in this work, showing **(a)** extinction coefficient spectrum and **(b)** fluorescence emission spectrum. QDs were purified and dispersed in hexane. A.U. = arbitrary units.

Table S1. FCS fitting parameters for QDs in BSA solutions

	p(NH_3^+)				p(COO^-)				p(OEG)				p(C_4ZW)				p(C_4ZW)$_1$(OEG)$_1$			
% BSA	D_1	f_1	D_2	f_2	D_1	f_1	D_2	f_2	D_1	f_1	D_2	f_2	D_1	f_1	D_2	f_2	D_1	f_1	D_2	f_2
0%	5.02	1	/	/	16.94	1	/	/	29.12	1	/	/	34.8	1	/	/	24.15	1	/	/
0.125%	0.27	1	/	/	13.62	0.85	5.67	0.15	16.94	1	/	/	13.62	0.97	3.43	0.03	22.3	0.90	9.9	0.10
0.25%	0.27	1	/	/	15.70	0.83	3.87	0.17	15.84	0.79	2.95	0.21	15.7	0.85	3.81	0.15	23.55	0.89	3.7	0.11
0.50%	0.15	1	/	/	12.54	0.60	4.39	0.40	12.54	0.58	4.21	0.42	12.54	0.72	4.39	0.28	15.46	0.81	5.6	0.19
1.00%	0.12	1	/	/	6.08	1	/	/	12.95	0.37	5.88	0.63	20.1	0.75	2.9	0.25	18.32	0.77	7.8	0.23

D is the diffusion coefficient in units of μm^2/s. f values are unitless.

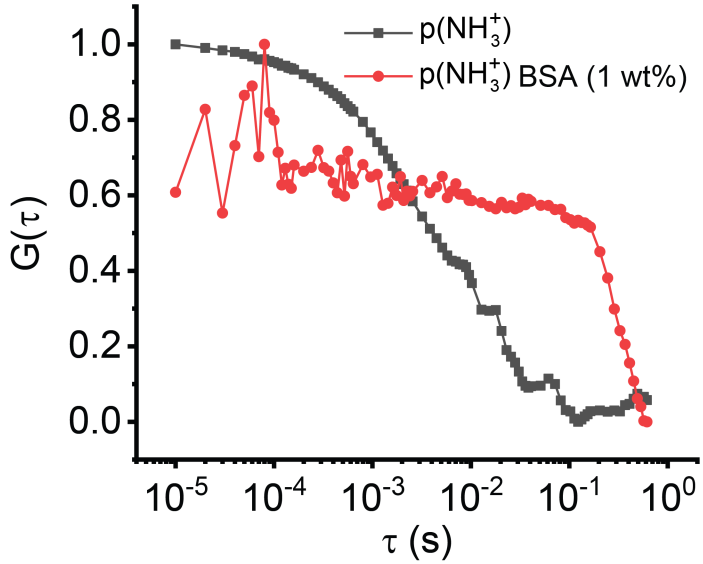

Figure S3. FCS autocorrelation curves of p(NH_3^+)-QDs in PBS (grey) or 1% BSA in PBS (red).

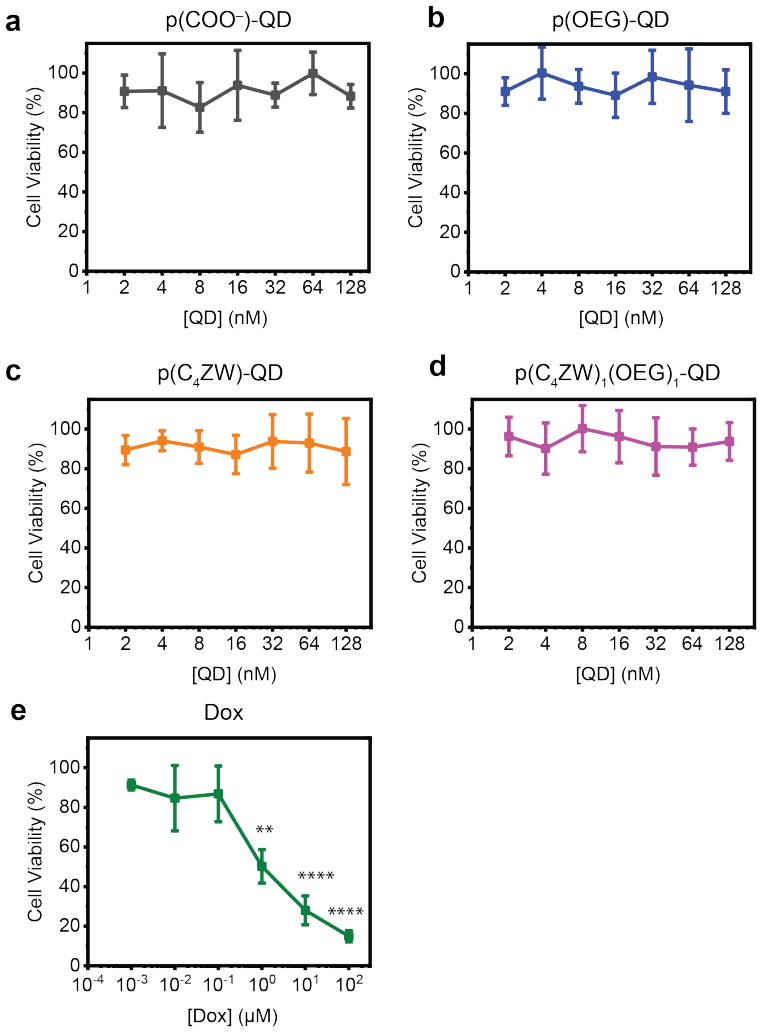

Figure S4. Cytotoxicity of QDs coated with four coatings on HeLa cells measured by the MTT assay, after 24 h of exposure. Panels show data for QDs coated with **(a)** p(COO^-), **(b)** p(OEG), **(c)** p(C_4ZW), and **(d)** p($C_4ZW)_1(OEG)_1$, as well as **(e)** doxorubicin, a cytotoxin used as a control. Values are reported as percent cell viability compared to cells treated with medium alone. Error bars indicate standard deviation from 3 biological replicates. Asterisks indicate *p*-values obtained by unpaired Student's t-test in comparison with non-treated cells (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$).

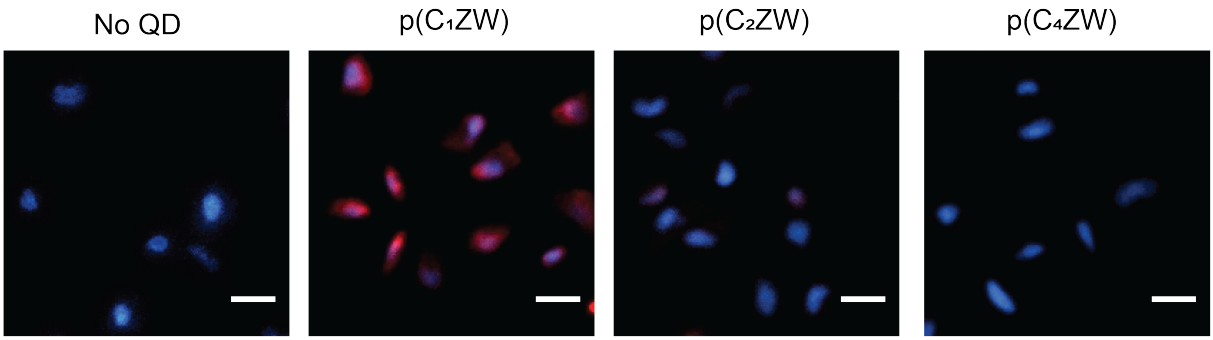

Figure S5. Nonspecific binding of p(C_nZW)-QDs to HeLa cells. Fluorescence micrographs show nuclear stain (blue) and QDs (red). Nonspecific binding is lowest for the p(C_4ZW)-QDs. Cells were imaged at 20×. Scale bars, 25 μm.

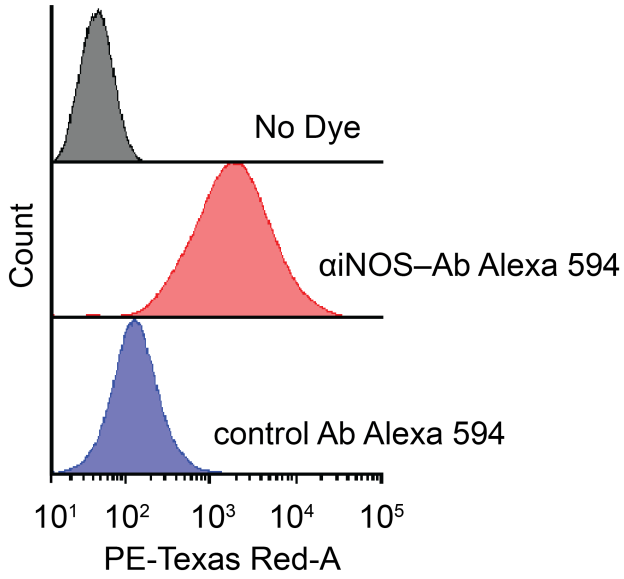

Figure S6. Dye control for intracellular labeling of iNOS measured through flow cytometry.

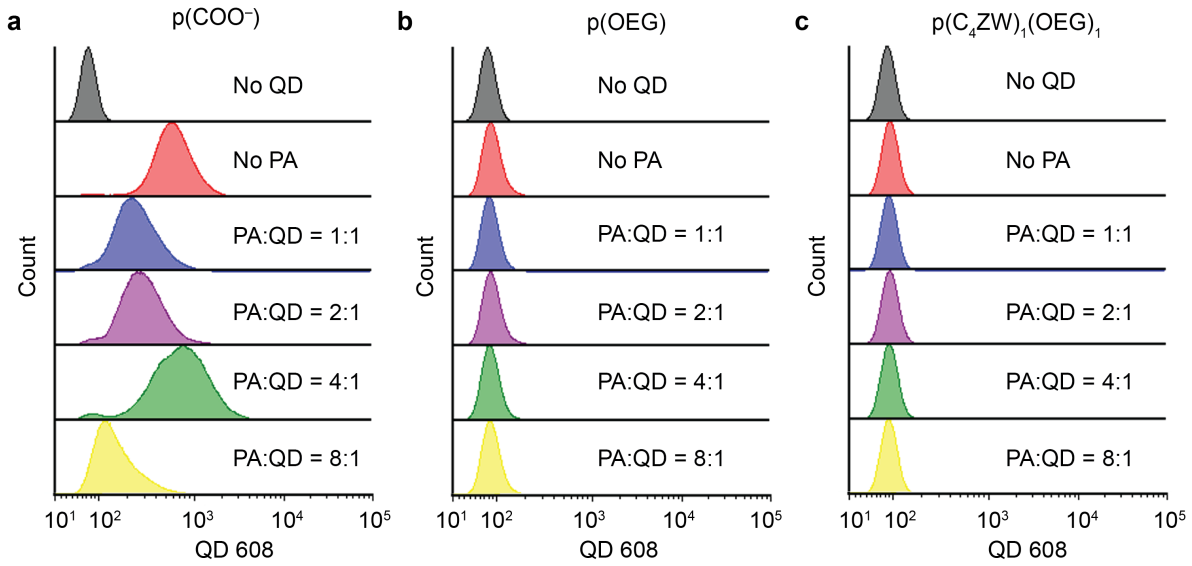

Figure S7. Nonspecific binding of QD-PA conjugates to fixed, permeabilized HeLa cells. Panels show representative fluorescence intensity histograms from flow cytometry of HeLa cells after 2-h room temperature incubation with QDs or QD-PA (40 nM) prepared using the indicated PA/QD ratios. **(a)** p(COO^-)-QD. **(b)** p(OEG)-QD. **(c)** p($C_4ZW)_1(OEG)_1$-QD.

Table S2. QD quantum yield (QY) with different ligand coatings

Ligand	**QY**
Oleylamine	57.0%*
$p(C_4ZW)_1(OEG)_1$	36.0%
Amphipol	31.5%
mPEG-SH	24.2%

* QY measured in hexane.

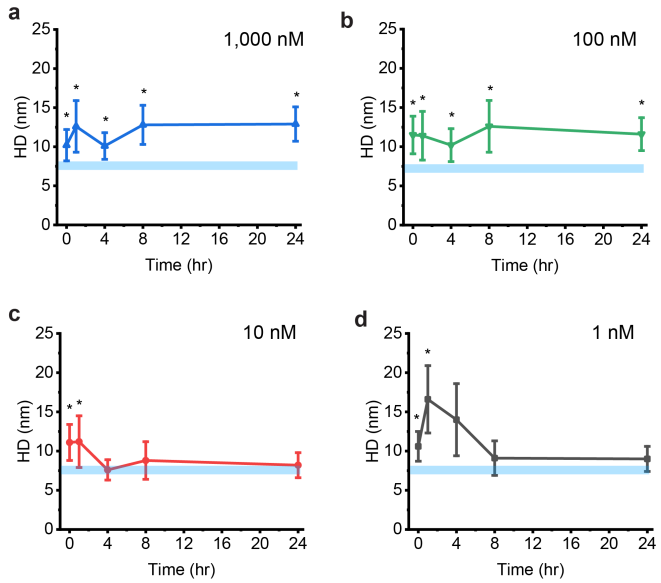

Figure S8. Stability of QD-PA conjugates measured by FCS. $p(C_4ZW)_1(OEG)_1$ QDs were conjugated to PA at a 4:1 PA/QD molar ratio at 1 μM QD concentration. The conjugates were then diluted to the indicated concentration and incubated for the indicated time in PBS. **(a)** 1000 nM. **(b)** 100 nM. **(c)** 10 nM. **(d)** 1 nM. FCS determined HD values are indicated as points with error bars representing standard deviations for 4 replicates. *p*-values from unpaired Student's t-test are indicated for comparison of each data point with that of the QDs alone without PA added. * $p < 0.05$. Comprehensive *p*-values in comparison with QD alone and 4:1 QD-PA are in **Table S3**.

Table S3. *p*-values for Student's t-test for comparisons of HD measured by FCS

	[QD-PA] = 1 nM		[QD-PA] = 10 nM		[QD-PA] = 100 nM		[QD-PA] = 1,000 nM	
Time	*vs* QD	*vs* QD-PA	*vs* QD	*vs* QD-PA	*vs* QD	*vs* QD-PA	*vs* QD	*vs* QD-PA
0 h	0.0477	0.6374	0.0460	0.8541	0.0449	0.5109	0.0482	1.0000
1 h	0.0406	0.1473	0.0445	0.9048	0.0202	0.6649	0.0481	0.9669
4 h	0.0781	0.4509	0.9166	0.0686	0.0305	0.4560	0.0326	0.5191
8 h	0.3429	0.2270	0.4451	0.2403	0.0118	0.5513	0.0416	0.6649
24 h	0.2681	0.1557	0.5778	0.1186	0.0191	0.4978	0.0301	0.9593

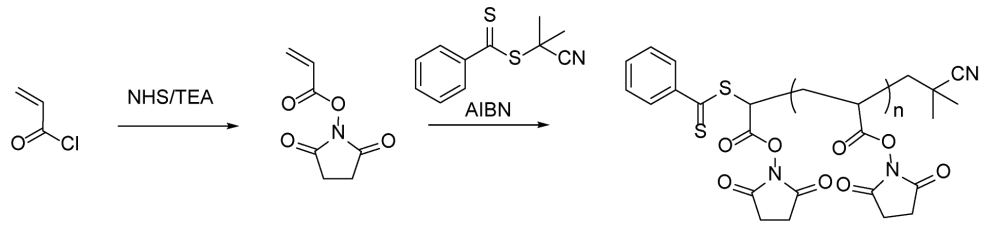

Figure S9. Synthesis scheme for PNAS polymer.

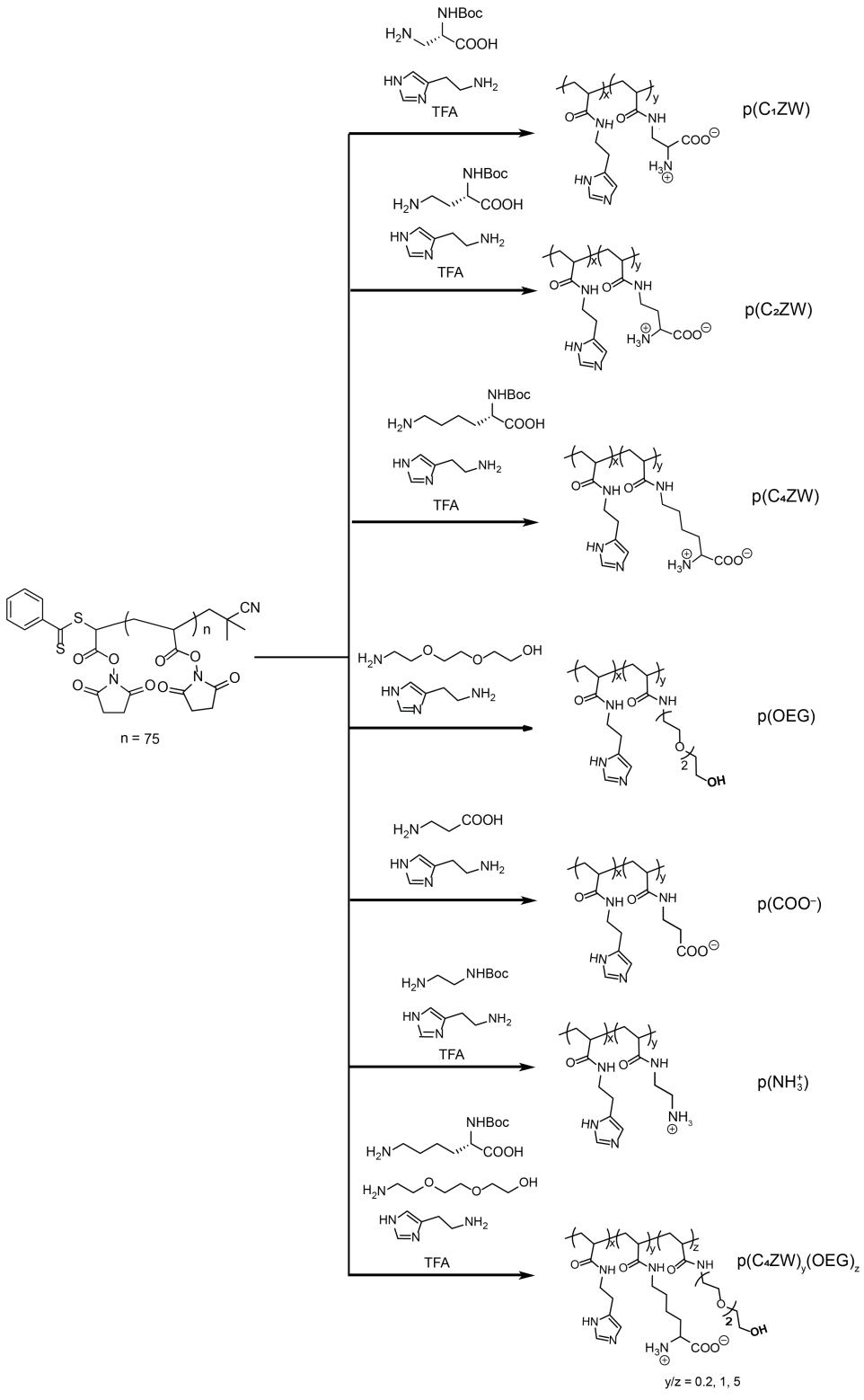

Figure S10. Synthesis scheme for multidentate polymers.

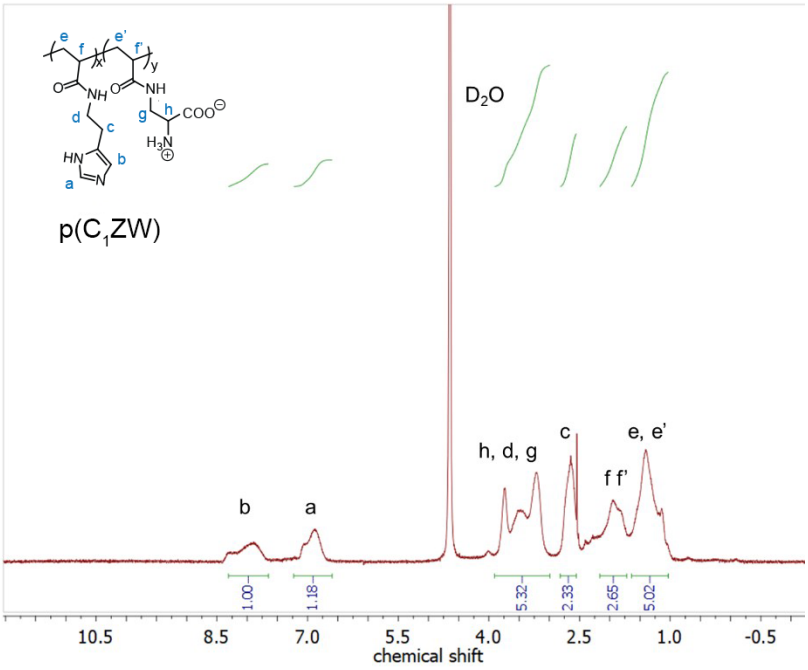

Figure S11. NMR spectrum of polymer p(C_1ZW)

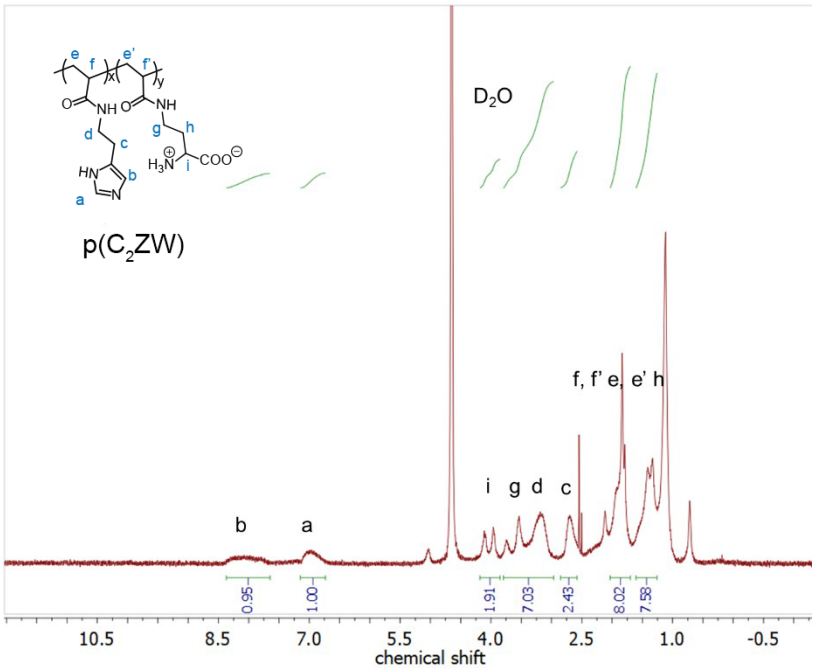

Figure S12. NMR spectrum of polymer p(C_2ZW)

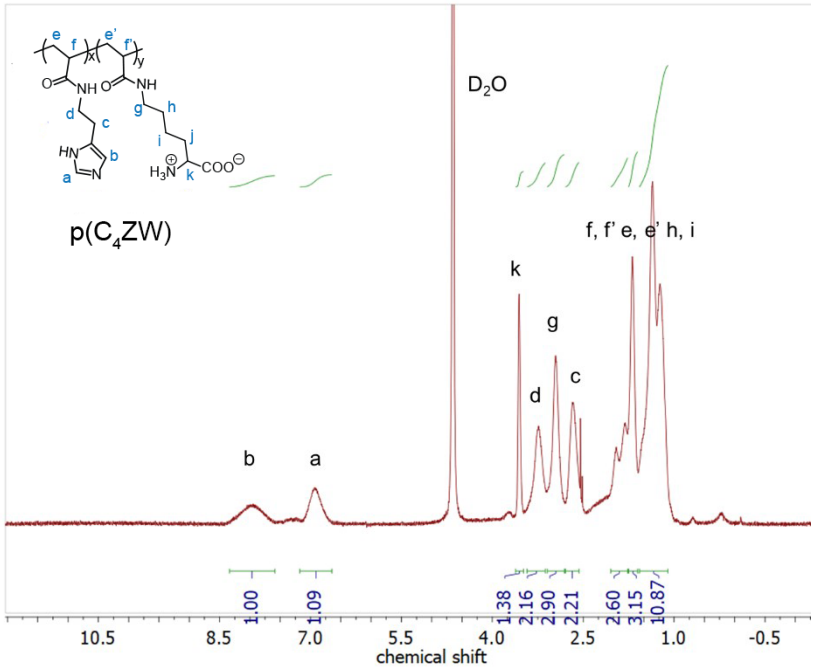

Figure S13. NMR spectrum of polymer p(C_4ZW)

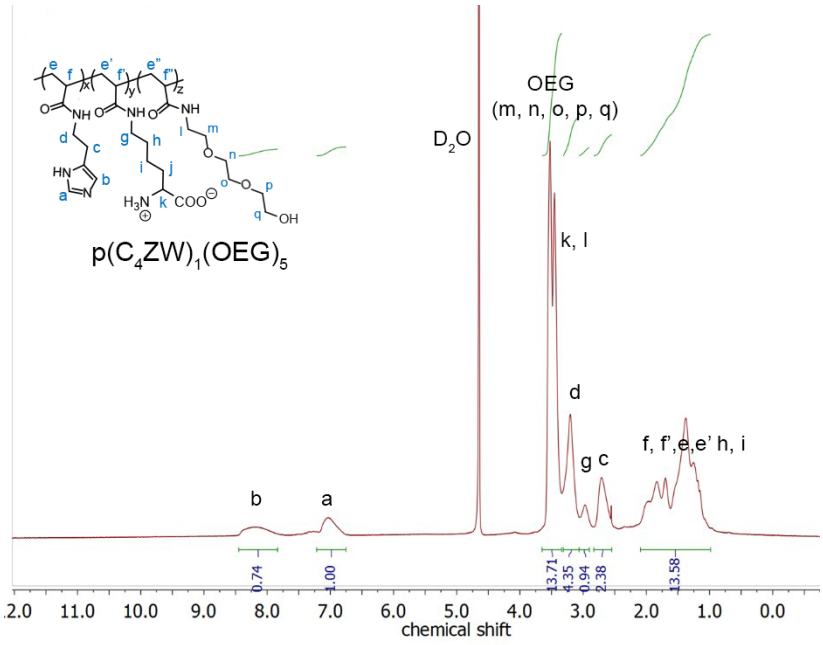

Figure S14. NMR spectrum of polymer p(C_4ZW)$_1$(OEG)$_5$

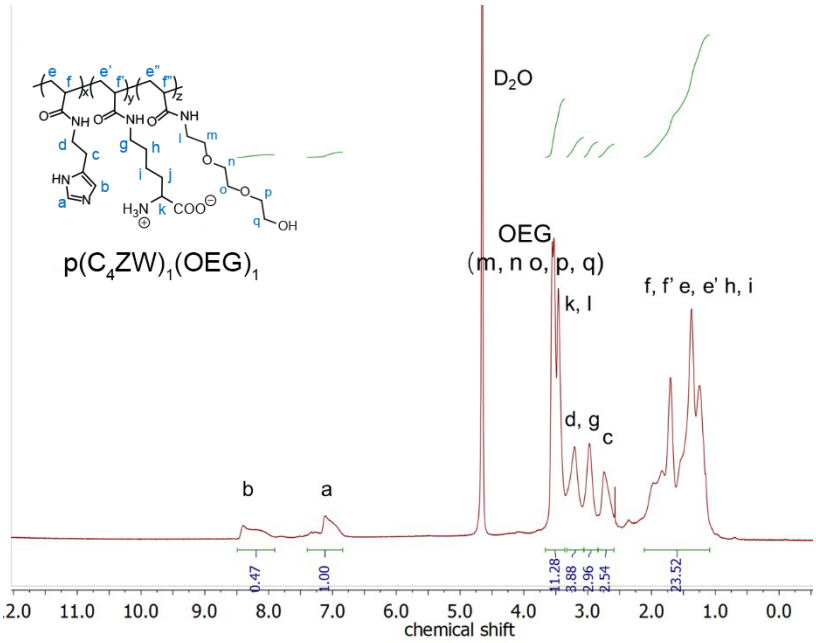

Figure S15. NMR spectrum of polymer $p(C_4ZW)_1(OEG)_1$

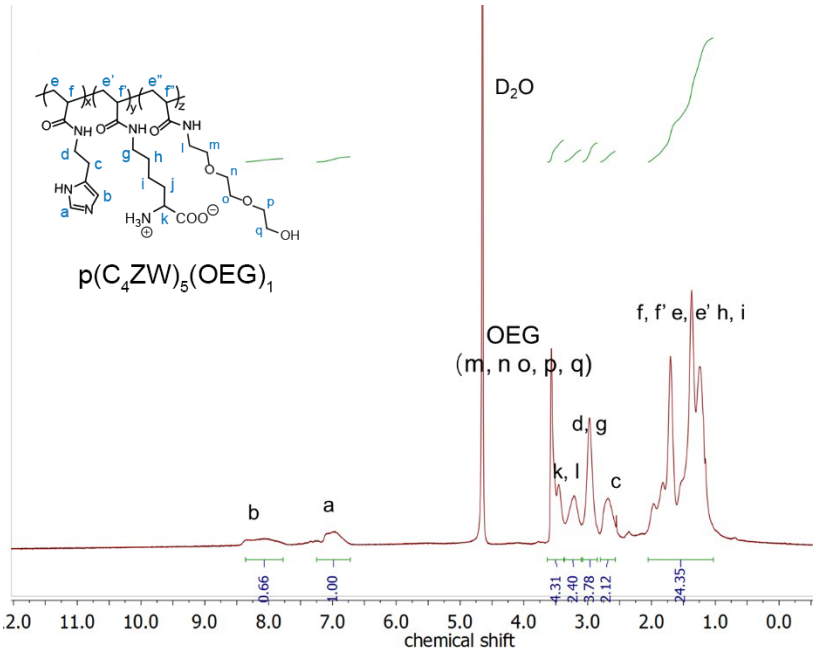

Figure S16. NMR spectrum of polymer $p(C_4ZW)_5(OEG)_1$

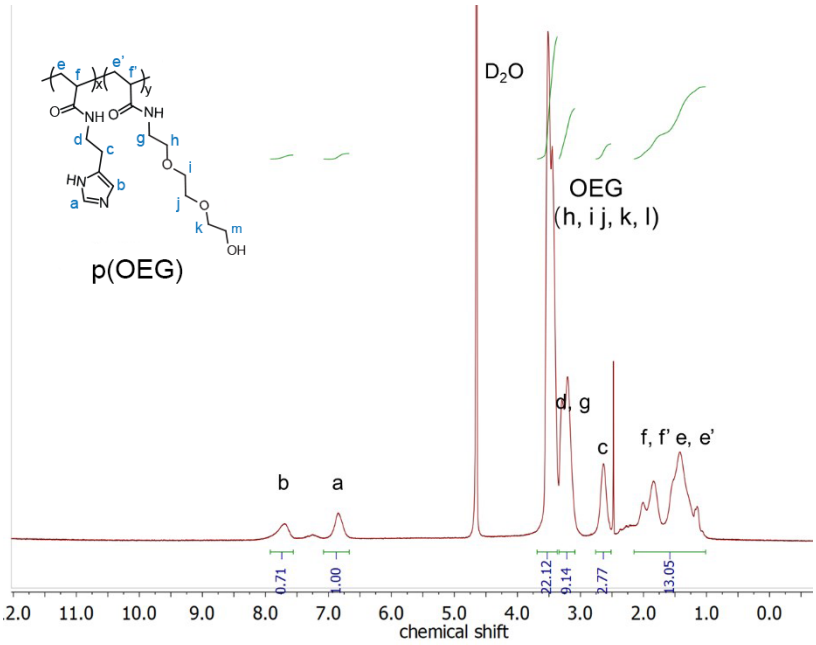

Figure S17. NMR spectrum of polymer p(OEG)

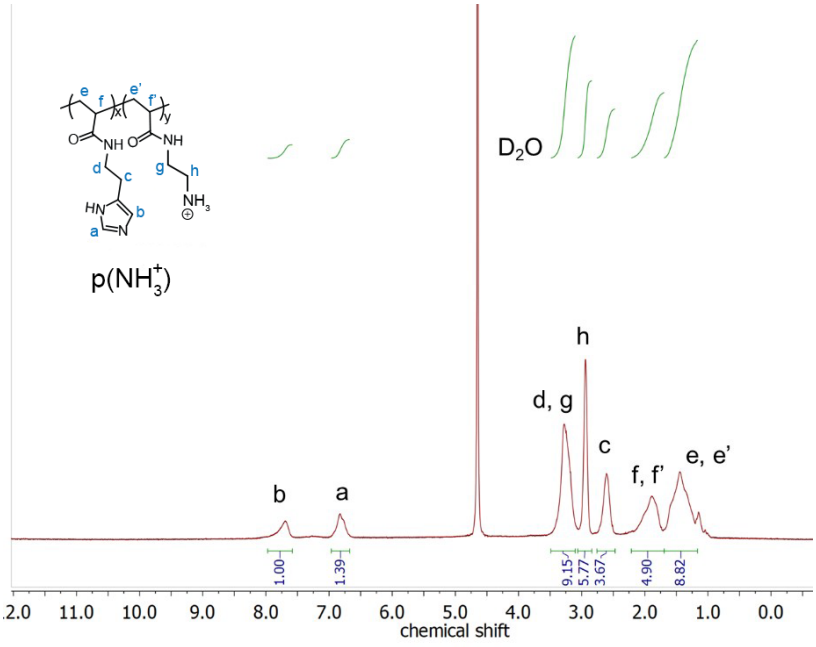

Figure S18. NMR spectrum of polymer p(NH_3^+)

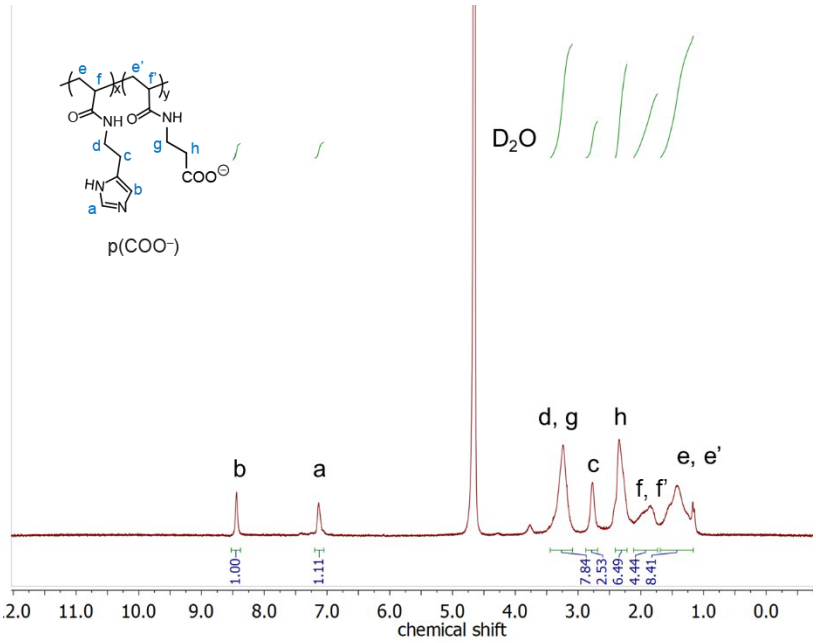

Figure S19. NMR spectrum of polymer p(COO^-)

3. Supporting References

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