

## Supporting Information

# Helical Poly(arginine) Mimics with Superior Cell-Penetrating and Molecular Transporting Properties

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### Materials and cells

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received unless otherwise specified. Anhydrous Tetrahydrofuran (THF), hexane and dimethylformamide (DMF) were dried by a column packed with 4Å molecular sieves and stored in a glove box. TAMRA-Arg9 and TAMRA-HIV-TAT were purchased from Biocompare (San Francisco, CA, USA). Plasmid DNA encoding luciferase (pCMV-Luc) was purchased from Elim Biopharmaceutics (Hayward, CA). TNF- $\alpha$  siRNA duplex and negative control siRNA containing scrambled sequences were supplied by Integrated DNA Technologies (Coralville, Iowa, USA) and dissolved in DEPC-treated water before use. The siRNA sequences were shown in Supplementary Table S1. FAM-labeled TNF- $\alpha$  siRNA duplex (FAM-siRNA) was used for in vitro cell uptake studies.

Raw264.7 (mouse monocyte macrophage), 3T3-L1 (mouse embryonic fibroblast), and HeLa (human cervix adenocarcinoma) cells were purchased from the American Type Culture Collection (Rockville, MD, USA), and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS).

### Instrumentation

$^1\text{H}$  and  $^{13}\text{C}\{^1\text{H}\}$  NMR spectra were recorded on a Varian UI400 MHz, a UI500NB MHz or a VXR-500 MHz spectrometer. Chemical shifts are reported in ppm and referenced to the solvent protio impurities and solvent  $^{13}\text{C}\{^1\text{H}\}$  resonances. Size exclusion chromatography (SEC) experiments were performed on a system equipped with an isocratic pump (Model 1100, Agilent Technology, Santa Clara, CA, USA), a DAWN HELEOS multi-angle laser light scattering detector (MALLS) detector, Wyatt Technology, Santa Barbara, CA, USA) and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The detection wavelength of HELEOS was set at 658 nm. Separations were performed using serially connected size exclusion columns (100 Å, 500 Å,  $10^3$  Å and  $10^4$  Å Phenogel columns, 5  $\mu\text{m}$ , 300  $\times$  7.8 mm, Phenomenex, Torrance, CA, USA) at 60 °C using DMF containing 0.1 M LiBr as the mobile phase. The MALLS detector is calibrated using pure toluene with no need for calibration using polymer standards and can be used for the determination of the absolute molecular weights (MWs). The MWs of polymers were determined based on the  $dn/dc$  value of each polymer

sample calculated offline by using the internal calibration system processed by the ASTRA V software (version 5.1.7.3, Wyatt Technology, Santa Barbara, CA, USA). The measured  $dn/dc$  of PCPLG, PCPDG, PCPDLG, PCHLG and PCOLG in DMF/0.1 M LiBr at 658 nm wavelength are 0.069, 0.075, 0.086, 0.102 and 0.085 mL/g respectively. Infrared spectra were recorded on a Perkin Elmer 100 serial FTIR spectrophotometer calibrated with polystyrene film. Circular dichroism (CD) measurements were carried out on a JASCO J-700 CD spectrometer. The polymer samples were prepared at concentrations of 0.05-0.5 mg/mL in general unless otherwise specified. The solution was placed in a quartz cell with a pathlength of 0.2 cm. The mean residue molar ellipticity of each polymer was calculated based on the measured apparent ellipticity by following the literature reported formulas: Ellipticity ( $[\theta]$  in  $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ ) = (millidegrees  $\times$  mean residue weight)/(pathlength in millimeters  $\times$  concentration of polypeptide in  $\text{mg ml}^{-1}$ ). The helicity of the polypeptides were calculated by the following equation: helicity =  $(-[\theta_{222}] + 3,000)/39,000$ .

### Synthesis of *N*-Pro-2-ynyl-guanidinium (PG)

Propargyl amine (0.12 g, 2 mmol), 1*H*-pyrazole-1-carboxamide hydrochloride (0.29 g, 2 mmol) and triethylamine (0.26 g, 2 mmol) were dissolved in DMF (1 mL), and the mixture was stirred at room temperature for 16 h. Then, the reaction solution was poured into 10-fold ethyl ether (10 mL) to remove the DMF and other impurities. Dry under vacuum to yield an oily product. Obtained 0.24 g (yield = 90%).  $^1\text{H NMR}$  [ $\text{D}_2\text{O}$ ,  $\delta$ , ppm]: 3.85 (s, 2H,  $\text{CH}\equiv\text{CH}_2$ -), 2.57 (s, 1H,  $\text{CH}\equiv\text{CH}_2$ -);  $^{13}\text{C}\{^1\text{H}\}$  NMR ( $\text{D}_2\text{O}$ ,  $\delta$ , ppm): 165.08, 77.91, 73.92 and 30.88; HR ESI-MS ( $m/z$ ) [ $\text{M}+\text{H}$ ] $^+$  calcd. for  $\text{C}_4\text{H}_8\text{N}_3$ , 98.0718; found 98.0721.

### Synthesis of Poly( $\gamma$ -3-azidopropanyl-L-glutamate) (PAPLG)

The polymer was synthesized by a literature procedure.  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ,  $\delta$ , ppm): 4.18 (s, 2H,  $\text{ClCH}_2\text{CH}_2\text{CH}_2$ -), 3.95 (br s, 1H,  $\text{CHNH}$ ), 3.40 (s, 2H,  $\text{ClCH}_2\text{CH}_2\text{CH}_2$ -), 2.68 (br s, 2H,  $-\text{COCH}_2\text{CH}_2$ -), 2.39 (br s, 2H,  $-\text{COCH}_2\text{CH}_2$ -), 1.92 (s, 2H,  $\text{ClCH}_2\text{CH}_2\text{CH}_2$ -).

### Synthesis of $\gamma$ -(3-Chloropropanyl)-D-glutamate, $\gamma$ -(6-Chlorohexyl)-L-glutamate and $\gamma$ -(8-Chlorooctyl)-L-glutamate

$\gamma$ -(3-Chloropropanyl)-D-glutamate was synthesized from a reported method. Obtained 10.0 g (yield = 66%).  $^1\text{H NMR}$  [ $\text{D}_2\text{O}$ ,  $\delta$ , ppm]: 4.13 (t, 2H,  $-\text{CH}_2\text{OOC}$ -), 3.61 (m, 1H,  $-\text{CHNH}_2$ ), 3.54 (t, 2H,  $-\text{CH}_2\text{Cl}$ ), 2.45 (t, 2H,  $-\text{CH}_2\text{COO}$ -), 1.97 (m, 4H,  $-\text{CH}_2\text{CH}_2\text{COO}$ - and  $\text{ClCH}_2\text{CH}_2\text{CH}_2\text{OOC}$ -);  $^{13}\text{C}\{^1\text{H}\}$  NMR ( $\text{D}_2\text{O}$ ,  $\delta$ , ppm): 174.42, 171.36, 62.65, 52.09, 41.80, 30.74, 29.70 and 24.88; HR ESI-MS ( $m/z$ ) [ $\text{M}+\text{H}$ ] $^+$  calcd. for  $\text{C}_8\text{H}_{15}\text{ClNO}_4$ , 244.0690; found 244.0690.

Typically, L-Glutamic acid (10.0 g, 68.0 mmol) and 6-chlorohexanol (15 mL) were mixed and stirred at 0  $^\circ\text{C}$ , followed by slowly adding  $\text{H}_2\text{SO}_4$  (4 mL). The reaction temperature allows warming up to room temperature after adding. Kept stirring for 16 h. Saturated  $\text{Na}_2\text{CO}_3$  solution was added to the reaction mixture and the product start to precipitate after the pH value close to 7. The raw product was collected by filtration and purified by recrystallization from isopropanol/ $\text{H}_2\text{O}$ . Obtained 10.0 g (yield = 55%).  $^1\text{H NMR}$  [ $\text{D}_2\text{O}/\text{DCl}(2\text{wt}\%)$ ,  $\delta$ , ppm]: 3.83 (m, 3H,  $-\text{CH}_2\text{OOC}$ - and  $-\text{CHNH}_2$ ), 3.32 (t, 2H,  $-\text{CH}_2\text{Cl}$ ), 2.35 (t, 2H,  $-\text{CH}_2\text{COO}$ -), 1.97 (m, 2H,  $-\text{CH}_2\text{CH}_2\text{COO}$ -), 1.47-1.11 (m, 8H,  $\text{ClCH}_2-(\text{CH}_2)_4-\text{CH}_2\text{OOC}$ -);  $^{13}\text{C}\{^1\text{H}\}$  NMR [ $\text{D}_2\text{O}/\text{DCl}(2\text{wt}\%)$ ,  $\delta$ , ppm]: 174.56, 171.32, 65.90, 52.10, 45.80, 31.81, 29.79, 27.63, 25.75, 24.95 and 24.48; HR ESI-MS ( $m/z$ ) [ $\text{M}+\text{H}$ ] $^+$  calcd. for  $\text{C}_{11}\text{H}_{21}\text{ClNO}_4$ , 266.1159; found 266.1158.

$\gamma$ -(8-Chlorooctyl)-L-glutamate was synthesized using the same method. Obtained 6.0 g (yield =

30%).  $^1\text{H}$  NMR [ $\text{D}_2\text{O}/\text{DCl}(2\text{wt}\%)$ ,  $\delta$ , ppm]: 3.83 (t, 2H,  $-\text{CH}_2\text{OOC}-$ ), 3.76 (m, 1H,  $-\text{CHNH}_2$ ), 3.23 (t, 2H,  $-\text{CH}_2\text{Cl}$ ), 2.33 (t, 2H,  $-\text{CH}_2\text{COO}-$ ), 1.94 (m, 2H,  $-\text{CH}_2\text{CH}_2\text{COO}-$ ), 1.45-1.03 (m, 12H,  $\text{ClCH}_2-(\text{CH}_2)_6-\text{CH}_2\text{OOC}-$ );  $^{13}\text{C}\{^1\text{H}\}$  NMR [ $\text{D}_2\text{O}/\text{DCl}(2\text{wt}\%)$ ,  $\delta$ , ppm]: 173.74, 171.16, 65.38, 52.10, 45.22, 32.59, 29.81, 29.12, 28.73, 28.36, 26.77, 25.71 and 25.09. HR ESI-MS ( $m/z$ ) [ $\text{M}+\text{H}$ ] $^+$  calcd. for  $\text{C}_{13}\text{H}_{25}\text{ClNO}_4$ , 294.1472; found 294.1472.

**Synthesis of  $\gamma$ -(3-Chloropropanyl)-D-glutamic Acid Based *N*-Carboxylanhydride (CP-D-NCA),  $\gamma$ -(3-Chloropropanyl)-D-glutamic Acid Based *N*-Carboxylanhydride (CP-DL-NCA),  $\gamma$ -(6-Chlorohexyl)-L-glutamic Acid Based *N*-Carboxylanhydride (CH-L-NCA) and  $\gamma$ -(8-Chlorooctyl)-L-glutamic Acid Based *N*-Carboxylanhydride (CO-L-NCA)**

A round-bottomed flask (100 mL) was charged with  $\gamma$ -(3-chloropropanyl)-D-glutamate (1.2 g, 5.4 mmol), phosgene (20%, 6 mL, 10.8 mmol, 2 equiv.) and anhydrous THF (30 mL) under nitrogen. The mixture was stirred at room temperature for 24 h over which period the  $\gamma$ -chlorohexanyl-L-glutamate was gradually dissolved. Removal of the solvent under vacuum yielded an oily liquid. Recrystallization by layering hexane on top of a  $\text{CH}_2\text{Cl}_2$  solution containing the oil was not successful, resulting in two separate liquid layers. The bottom layer was separated and subjected to vacuum to give a clear liquid. Obtained 0.72 g (yield = 54%).  $^1\text{H}$  NMR [ $\text{CDCl}_3$ ,  $\delta$ , ppm]: 6.55 (s, 1H,  $-\text{NH}$ ), 4.42 (t, 1H,  $-\text{CHNH}$ ), 4.27 (t, 2H,  $-\text{CH}_2\text{OOC}-$ ), 3.62 (t, 2H,  $-\text{CH}_2\text{Cl}$ ), 2.57 (t, 2H,  $-\text{CH}_2\text{CH}_2\text{COO}-$ ), 2.10-2.40 (m, 4H,  $-\text{CH}_2\text{CH}_2\text{COO}-$  and  $\text{ClCH}_2\text{CH}_2\text{CH}_2\text{OOC}-$ );  $^{13}\text{C}\{^1\text{H}\}$  NMR [ $\text{CDCl}_3$ ,  $\delta$ , ppm]: 172.70, 169.53, 151.92, 62.23, 57.15, 41.25, 31.46, 29.87 and 27.04. HR ESI-MS ( $m/z$ ) [ $\text{M}+\text{H}$ ] $^+$  calcd. for  $\text{C}_9\text{H}_{13}\text{ClNO}_5$ , 250.0482; found 250.0488.

$\gamma$ -(6-Chlorohexyl)-L-glutamic Acid Based *N*-Carboxylanhydride (CH-L-NCA) was synthesized using the same method. Obtained 1.0 g (yield = 61%).  $^1\text{H}$  NMR [ $\text{CDCl}_3$ ,  $\delta$ , ppm]: 6.69 (s, 1H,  $-\text{NH}$ ), 4.41 (t, 1H,  $-\text{CHNH}$ ), 4.10 (t, 2H,  $-\text{CH}_2\text{OOC}-$ ), 3.54 (t, 2H,  $-\text{CH}_2\text{Cl}$ ), 2.55 (t, 2H,  $-\text{CH}_2\text{CH}_2\text{COO}-$ ), 2.13-2.26 (m, 2H,  $-\text{CH}_2\text{CH}_2\text{COO}-$ ), 1.38-1.78 (m, 8H,  $\text{ClCH}_2-(\text{CH}_2)_4-\text{CH}_2\text{OOC}-$ );  $^{13}\text{C}\{^1\text{H}\}$  NMR [ $\text{CDCl}_3$ ,  $\delta$ , ppm]: 172.93, 169.69, 152.02, 65.47, 57.26, 45.18, 32.58, 30.05, 28.55, 27.14, 26.61 and 25.43. HR ESI-MS ( $m/z$ ) [ $\text{M}+\text{H}$ ] $^+$  calcd. for  $\text{C}_{12}\text{H}_{19}\text{ClNO}_5$ , 292.0952; found 292.0957.

$\gamma$ -(8-Chlorooctyl)-L-glutamic acid-based *N*-carboxylanhydride (CO-L-NCA) was synthesized using the same method. Obtained 0.5 g (yield = 31%).  $^1\text{H}$  NMR [ $\text{CDCl}_3$ ,  $\delta$ , ppm]: 6.65 (s, 1H,  $-\text{NH}$ ), 4.40 (t, 1H,  $-\text{CHNH}$ ), 4.09 (t, 2H,  $-\text{CH}_2\text{OOC}-$ ), 3.53 (t, 2H,  $-\text{CH}_2\text{Cl}$ ), 2.55 (t, 2H,  $-\text{CH}_2\text{CH}_2\text{COO}-$ ), 2.12-2.26 (m, 2H,  $-\text{CH}_2\text{CH}_2\text{COO}-$ ), 1.32-1.77 (m, 12H,  $\text{ClCH}_2-(\text{CH}_2)_6-\text{CH}_2\text{OOC}-$ );  $^{13}\text{C}\{^1\text{H}\}$  NMR [ $\text{CDCl}_3$ ,  $\delta$ , ppm]: 172.95, 169.71, 152.01, 65.66, 57.27, 45.38, 32.76, 30.08, 29.24, 28.93, 28.66, 27.16, 26.96 and 25.96. HR ESI-MS ( $m/z$ ) [ $\text{M}+\text{H}$ ] $^+$  calcd. for  $\text{C}_{14}\text{H}_{23}\text{ClNO}_5$ , 320.1265; found 320.1269.

**Synthesis of Poly( $\gamma$ -3-chloropropanyl-D-glutamate) (PCPDG), Poly( $\gamma$ -3-chloropropanyl-DL-glutamate) (PCPDLG), Poly( $\gamma$ -6-chlorohexyl-L-glutamate) (PCHLG) and Poly( $\gamma$ -8-chlorooctyl-L-glutamate) (PCOLG)**

Typically, inside a glove-box, CP-D-NCA (100 mg, 0.4 mmol) was dissolved in DMF (1.0 mL), followed by adding the HMDS/DMF solution (80.3  $\mu\text{L}$ , 0.1 M, M/I=50). The reaction mixture was stirred for 48 h at room temperature. The polymer was precipitated from cold methanol and collected by centrifuge. The product was dried under vacuum at 40  $^\circ\text{C}$  for 8 h. Obtained 55 mg (yield = 67%).  $^1\text{H}$  NMR [ $\text{CDCl}_3/\text{TFA}-d$  ( $v/v = 85/15$ ),  $\delta$ , ppm]: 4.54 (s, 1H,  $-\text{CHNH}$ ), 4.28 (s, 2H,  $-\text{CH}_2\text{OOC}-$ ), 3.59 (s, 2H,  $-\text{CH}_2\text{Cl}$ ), 2.50 (s, 2H,  $-\text{CH}_2\text{CH}_2\text{COO}-$ ), 1.95-2.40

(m, 4H,  $-CH_2CH_2COO-$  and  $ClCH_2CH_2CH_2OOC-$ );  $^{13}C\{^1H\}$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 175.43, 173.13, 63.23, 53.45, 40.77, 30.99, 30.17 and 26.97.

Poly( $\gamma$ -3-chloropropanyl-DL-glutamate) was synthesized using the same method by adding equivalent CP-L-NCA and CP-D-NCA. Obtained 50 mg (yield = 60%).  $^1H$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 4.61 (s, 1H,  $-CHNH$ ), 4.29 (s, 2H,  $-CH_2OOC-$ ), 3.59 (s, 2H,  $-CH_2Cl$ ), 2.54 (s, 2H,  $-CH_2CH_2COO-$ ), 1.95-2.40 (m, 4H,  $-CH_2CH_2COO-$  and  $ClCH_2CH_2CH_2OOC-$ );  $^{13}C\{^1H\}$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 175.51, 173.15, 63.26, 53.44, 40.74, 30.97, 30.13 and 26.96.

Poly( $\gamma$ -6-chlorohexyl-L-glutamate) was synthesized using the same method. Obtained 61 mg (yield = 70%).  $^1H$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 4.55 (s, 1H,  $-CHNH$ ), 4.08 (s, 2H,  $-CH_2OOC-$ ), 3.52 (s, 2H,  $-CH_2Cl$ ), 2.48 (s, 2H,  $-CH_2CH_2COO-$ ), 2.15-2.48 (d, 2H,  $-CH_2CH_2COO-$ ), 1.34-1.98 (m, 8H,  $ClCH_2-(CH_2)_4-CH_2OOC-$ );  $^{13}C\{^1H\}$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 176.05, 173.25, 66.79, 53.41, 44.85, 32.31, 30.21, 27.99, 27.15, 26.38, 24.94.

Poly( $\gamma$ -8-chlorooctyl-L-glutamate) was synthesized using the same method. Obtained 56 mg (yield = 65%).  $^1H$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 4.56 (s, 1H,  $-CHNH$ ), 4.07 (s, 2H,  $-CH_2OOC-$ ), 3.53 (s, 2H,  $-CH_2Cl$ ), 2.48 (s, 2H,  $-CH_2CH_2COO-$ ), 1.98-2.14 (d, 2H,  $-CH_2CH_2COO-$ ), 1.30-1.76 (m, 12H,  $ClCH_2-(CH_2)_6-CH_2OOC-$ );  $^{13}C\{^1H\}$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 176.13, 173.29, 67.02, 53.39, 45.17, 32.59, 30.20, 29.04, 28.73, 27.13, 26.79 and 25.51.

### **Synthesis of Poly( $\gamma$ -3-azidopropanyl-D-glutamate) (PAPDG), Poly( $\gamma$ -3-azidopropanyl-DL-glutamate) (PAPDLG), Poly( $\gamma$ -6-azidohexyl-L-glutamate) (PAHLG) and Poly( $\gamma$ -8-azidooctyl-L-glutamate) (PAOLG)**

A DMF (2 mL) solution of PCPDG (41 mg, 0.2 mmol of chloro groups) and sodium azide (0.16 g, 2.4 mmol) was stirred at 60 °C for 48 h and allowed to cool to room temperature. The reaction mixture was filtered to remove any inorganic salts. DMF was removed by vacuum distillation at 60 °C to yield a polymer film which was further purified by dissolution in  $CHCl_3$ , filtration and remove the solvent. The resulting polymer was collected by filtration and dried at under vacuum at 40 °C for 8 h. Obtained 32 mg (yield = 75%).  $^1H$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 4.56 (s, 1H,  $-CHNH$ ), 4.20 (s, 2H,  $-CH_2OOC-$ ), 3.40 (s, 2H,  $-CH_2N_3$ ), 2.51 (s, 2H,  $-CH_2CH_2COO-$ ), 1.90-2.20 (m, 4H,  $-CH_2CH_2COO-$  and  $-COOCH_2CH_2CH_2N_3$ );  $^{13}C\{^1H\}$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 175.39, 173.20, 63.36, 53.62, 48.10, 30.19, 27.58 and 26.92.

Poly( $\gamma$ -3-azidopropanyl-DL-glutamate) was synthesized using the same method. Obtained 30 mg (yield = 70%).  $^1H$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 4.55 (s, 1H,  $-CHNH$ ), 4.18 (s, 2H,  $-CH_2OOC-$ ), 3.38 (s, 2H,  $-CH_2N_3$ ), 2.48 (s, 2H,  $-CH_2CH_2COO-$ ), 1.90-2.20 (m, 4H,  $-CH_2CH_2COO-$  and  $-COOCH_2CH_2CH_2N_3$ );  $^{13}C\{^1H\}$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 175.36, 173.05, 63.34, 53.56, 48.09, 30.13, 27.58 and 26.85.

Poly( $\gamma$ -6-azidohexyl-L-glutamate) was synthesized using the same method. Obtained 38 mg (yield = 75%).  $^1H$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 4.61 (s, 1H,  $-CHNH$ ), 4.12 (s, 2H,  $-CH_2OOC-$ ), 3.33 (s, 2H,  $-CH_2N_3$ ), 2.53 (s, 2H,  $-CH_2CH_2COO-$ ), 1.99-2.17 (d, 2H,  $-CH_2CH_2COO-$ ), 1.40-1.66 (m, 8H,  $-COOCH_2-(CH_2)_4-CH_2N_3$ );  $^{13}C\{^1H\}$  NMR [ $CDCl_3/TFA-d$  (v/v = 85/15),  $\delta$ , ppm]: 175.88, 173.22, 66.57, 53.43, 51.44, 30.19, 28.52, 28.05, 27.11, 26.26 and 25.25.

Poly( $\gamma$ -8-azidooctyl-L-glutamate) was synthesized using the same method. Obtained 40 mg

(yield = 80%).  $^1\text{H}$  NMR [ $\text{CDCl}_3/\text{TFA-}d$  (v/v = 85/15),  $\delta$ , ppm]: 4.62 (s, 1H,  $-\text{CHNH}$ ), 4.11 (s, 2H,  $-\text{CH}_2\text{OOC}-$ ), 3.35 (s, 2H,  $-\text{CH}_2\text{N}_3$ ), 2.53 (s, 2H,  $-\text{CH}_2\text{CH}_2\text{COO}-$ ), 2.00-2.18 (d, 2H,  $-\text{CH}_2\text{CH}_2\text{COO}-$ ), 1.33-1.65 (m, 12H,  $-\text{COOCH}_2-(\text{CH}_2)_6-\text{CH}_2\text{N}_3$ );  $^{13}\text{C}\{^1\text{H}\}$  NMR [ $\text{CDCl}_3/\text{TFA-}d$  (v/v = 85/15),  $\delta$ , ppm]: 175.81, 173.16, 66.75, 53.38, 51.59, 30.18, 29.04, 28.99, 28.66, 18.17, 27.08, 26.60 and 25.54.

### Synthesis of Guanidinium-rich Polypeptides (P1-P14)

Typically, inside a glove-box, *N*-pro-2-ynyl-guanidinium (10 mg, 0.1 mmol), PAPLG (21.2 mg, 0.1 mmol of the functional groups), PMDETA (70  $\mu\text{L}$ , 0.5 mmol) were dissolved in DMF (1 mL), followed by adding the above solution to a vial with CuBr (14 mg, 0.1 mmol). The reaction solution was stirred at room temperature for 24 h. The reaction was quenched by exposing to air and 1N  $\text{HCl}_{\text{aq}}$  was added to the reaction solution until it turned to yellow. The resulting polymer was purified by dialysis against DI water for three days in a dialysis membrane tube with a cutoff molecular weight of 1000  $\text{g}\cdot\text{mol}^{-1}$ . White solid product was obtained after freeze-dry (yield = 60% - 70%).

**P1, P4:**  $^1\text{H}$  NMR [ $\text{D}_2\text{O}$ ,  $\delta$ , ppm]: 7.89 (s, 1H, triazole), 4.35 (s, 4H,  $-\text{COOCH}_2-$  and triazole- $\text{CH}_2\text{NH}-$ ), 4.11 (br, 1H,  $-\text{CHNH}$ ), 3.88 (s, 2H,  $-\text{COOCH}_2\text{CH}_2\text{CH}_2-$ ), 1.83-2.28 (m, 6H,  $-\text{CH}_2\text{CH}_2\text{COOCH}_2\text{CH}_2-$ ).

**P2:**  $^1\text{H}$  NMR [ $\text{D}_2\text{O}$ ,  $\delta$ , ppm]: 7.86 (s, 1H, triazole), 4.33 (s, 4H,  $-\text{COOCH}_2-$  and triazole- $\text{CH}_2\text{NH}-$ ), 3.95 (br, 1H,  $-\text{CHNH}$ ), 3.87 (s, 2H,  $-\text{COOCH}_2\text{CH}_2\text{CH}_2-$ ), 1.83-2.28 (m, 6H,  $-\text{CH}_2\text{CH}_2\text{COOCH}_2\text{CH}_2-$ ).

**P3:**  $^1\text{H}$  NMR [ $\text{D}_2\text{O}$ ,  $\delta$ , ppm]: 7.89 (s, 1H, triazole), 4.32 (s, 4H,  $-\text{COOCH}_2-$  and triazole- $\text{CH}_2\text{NH}-$ ), 4.07 (br, 1H,  $-\text{CHNH}$ ), 3.80 (s, 2H,  $-\text{COOCH}_2\text{CH}_2\text{CH}_2-$ ), 1.72-2.17 (m, 6H,  $-\text{CH}_2\text{CH}_2\text{COOCH}_2\text{CH}_2-$ ).

**P5:**  $^1\text{H}$  NMR [ $\text{D}_2\text{O}$ ,  $\delta$ , ppm]: 7.87 (s, 1H, triazole), 4.35 (s, 2H,  $-\text{COOCH}_2-$ ), 4.21 (s, 2H, triazole- $\text{CH}_2\text{NH}-$ ), 3.85 (br, 3H,  $-\text{CHNH}$  and  $-\text{COOCH}_2\text{CH}_2\text{CH}_2-$ ), 2.00-2.46 (m, 4H,  $-\text{CH}_2\text{CH}_2\text{COO}-$ ), 1.11-1.67 (m, 8H,  $\text{ClCH}_2-(\text{CH}_2)_4-\text{CH}_2\text{OOC}-$ ).

**P6-P8:**  $^1\text{H}$  NMR [ $\text{D}_2\text{O}$ ,  $\delta$ , ppm]: 7.88 (s, 1H, triazole), 4.35 (s, 2H,  $-\text{COOCH}_2-$ ), 4.17 (s, 2H, triazole- $\text{CH}_2\text{NH}-$ ), 3.84 (br, 3H,  $-\text{CHNH}$  and  $-\text{COOCH}_2\text{CH}_2\text{CH}_2-$ ), 2.02-2.51 (m, 4H,  $-\text{CH}_2\text{CH}_2\text{COO}-$ ), 1.05-1.65 (m, 12H,  $\text{ClCH}_2-(\text{CH}_2)_6-\text{CH}_2\text{OOC}-$ ).

**P9:**  $^1\text{H}$  NMR [ $\text{D}_2\text{O}$ ,  $\delta$ , ppm]: 7.87 (s, 1H, triazole), 4.35 (br, 4H,  $-\text{COOCH}_2-$  and triazole- $\text{CH}_2\text{NH}-$ ), 3.89 (br, 3H,  $-\text{CHNH}$  and  $-\text{COOCH}_2\text{CH}_2\text{CH}_2-$ ), 1.60-2.60 (m, 8H,  $-\text{CH}_2\text{CH}_2\text{COOCH}_2\text{CH}_2-$  and  $\text{CH}_3\text{CH}_2\text{CH}_2-$ ), 0.64-1.36 (m, 5H,  $\text{CH}_3\text{CH}_2\text{CH}_2-$ ).

**P10:**  $^1\text{H}$  NMR [ $\text{D}_2\text{O}$ ,  $\delta$ , ppm]: 7.86 (s, 1H, triazole), 4.35 (br, 4H,  $-\text{COOCH}_2-$  and triazole- $\text{CH}_2\text{NH}-$ ), 3.89 (br, 3H,  $-\text{CHNH}$  and  $-\text{COOCH}_2\text{CH}_2\text{CH}_2-$ ), 1.60-2.60 (m, 8H,  $-\text{CH}_2\text{CH}_2\text{COOCH}_2\text{CH}_2-$  and  $\text{CH}_3(\text{CH}_2)_2\text{CH}_2-$ ), 0.62-1.32 (m, 7H,  $\text{CH}_3(\text{CH}_2)_2\text{CH}_2-$ ).

**P11, P13-P14:**  $^1\text{H}$  NMR [ $\text{D}_2\text{O}$ ,  $\delta$ , ppm]: 7.87 (s, 1H, triazole), 4.31 (br, 4H,  $-\text{COOCH}_2-$  and triazole- $\text{CH}_2\text{NH}-$ ), 3.85 (br, 3H,  $-\text{CHNH}$  and  $-\text{COOCH}_2\text{CH}_2\text{CH}_2-$ ), 1.80-2.60 (m, 8H,  $-\text{CH}_2\text{CH}_2\text{COOCH}_2\text{CH}_2-$  and  $\text{CH}_3(\text{CH}_2)_3\text{CH}_2-$ ), 0.57-1.41 (m, 9H,  $\text{CH}_3(\text{CH}_2)_3\text{CH}_2-$ ).

**P12:**  $^1\text{H}$  NMR [ $\text{D}_2\text{O}$ ,  $\delta$ , ppm]: 7.87 (s, 1H, triazole), 4.36 (br, 4H,  $-\text{COOCH}_2-$  and triazole- $\text{CH}_2\text{NH}-$ ), 3.89 (br, 3H,  $-\text{CHNH}$  and  $-\text{COOCH}_2\text{CH}_2\text{CH}_2-$ ), 1.60-2.60 (m, 8H,  $-\text{CH}_2\text{CH}_2\text{COOCH}_2\text{CH}_2-$  and  $\text{CH}_3(\text{CH}_2)_4\text{CH}_2-$ ), 0.57-1.34 (m, 11H,  $\text{CH}_3(\text{CH}_2)_4\text{CH}_2-$ ).

### Synthesis of Guanidinium-rich RhB-polypeptides Conjugate

Typically, guanidinium-rich polypeptide (23 mg, 0.67 mmol of repeating units,  $3.3\times 10^{-6}$  mol of primary amine) was dissolved in  $\text{NaHCO}_3$  solution (2.5 mL, 0.2 M). RhB-NCS (2 mg) was

dissolved in DMSO (0.2 mL) and added to the above aqueous solution. The reaction vial was wrapped with aluminum foil. The reaction was stirring at room temperature for 12 h, followed by dialysis against DI water (the beaker was wrapped with aluminum foil) for three days in a dialysis membrane tube with a cutoff molecular weight of 1000 g·mol<sup>-1</sup>. Red solid product was obtained after freeze-dry (yield = 60%~70%).

### **Cell penetration of guanidinium-rich polypeptides**

Cells were seeded on 96-well plates at  $1 \times 10^4$  cells/well and cultured for 24 h before they reached confluence. The medium was refreshed with serum-free DMEM, and RhB-polypeptides were added at 2 µg/well. TAMRA-Arg9 and TAMRA-HIV-TAT were used as controls. After incubation at 37 °C for 2 h, the cells were washed with cold PBS containing 20 U/mL heparin for 3 times, which could completely remove surface-bound cationic proteins from cells. Cells were then lysed with the RIPA lysis buffer at RT for 20 min before assessment of the RhB-polypeptide content by spectrofluorimetry and protein level using the BCA kit. The uptake level was expressed as µg RhB-polypeptide associated with 1 mg of cellular protein.

To visualize the cell penetration and intracellular distribution of polypeptides, cells were allowed to grow on coverslips in 6-well plate, onto which RhB-P14 was added at 5 µg/mL. Following incubation at 37 °C or 4 °C for 2 h, cells were washed with heparin-containing PBS for 3 times before observation with CLSM.

### **Mechanistic probe into the cell penetration of guanidinium-rich polypeptides**

To explore the mechanism involved in the penetration of polypeptides, cells were preincubated with endocytosis inhibitors including chlorpromazine (10 µg/mL), genistein (200 µg/mL), methyl-β-cyclodextrin (50 µM), and wortmannin (50 nM) for 30 min prior to addition of the polypeptides and throughout the 2-h uptake experiment at 37 °C. To block the energy-dependent endocytosis, uptake study was performed at 4 °C. The cell uptake level was determined as described above, and results were expressed as percentage uptake of the control where cells were incubated with RhB-P14 at 37 °C for 2 h. To further explore the caveolae-mediated endocytosis, HeLa cells were incubated with RhB-P14 and FITC-CTB (5 µg/mL) for 1 h before CLSM observation. To evaluate the clathrin-mediated pathway, cells were incubated with RhB-P14 and transferrin-Alexa Fluor 647 (10 µg/mL) for 1 h before CLSM observation.

Polypeptide-induced pore formation on cell membranes was evaluated in terms of the cell uptake of membrane-impermeable FITC. Briefly, HeLa cells were seeded on 96-well plates at  $1 \times 10^4$  cells/well and cultured for 24 h. The medium was replaced with serum-free DMEM, and polypeptides and FITC were added at 2 µg/well and 0.2 µg/well, respectively. Cells treated with only FITC served as the control. Following incubation at 37 °C for 2 h, cells were washed with heparin-containing cold PBS for 3 times and lysed with the RIPA lysis buffer. The uptake level of FITC in the lysate was quantified by spectrofluorimetry, while the protein level was determined using the BCA kit. Results were expressed as µg FITC associated with 1 mg of cellular protein.

### **Cytotoxicity**

Cells were seeded on 96-well plate at  $1 \times 10^4$  cells/well and cultured for 24 h before refreshment of the media with serum-free DMEM (100 µL/well). Polypeptides were added to each well at 10, 20, 50, and 100 µg/mL, respectively. Following incubation for 4 h, the cell viability was evaluated by the MTT assay.

### **Preparation of P14/DNA and P14/siRNA complexes**

P14, pCMV-Luc, and TNF- $\alpha$  siRNA were separately dissolved in DEPC water at 0.2 mg/mL. P14 was added to the pCMV-Luc or TNF- $\alpha$  siRNA solution at various N/P ratios, and the mixture was vortexed for 5 s and incubated at 37 °C for 30 min to allow complexation. The condensation of DNA or siRNA by P14 was evaluated by the gel retardation assay after electrophoresis (DNA: 1% agarose gel, 100 V, 30 min; siRNA: 4% agarose gel, 56V, 40 min).

### **Flow cytometry**

DNA (1 mg/mL) was labeled with YOYO-1 (20  $\mu$ M), an intercalating dye, at one dye molecule per 50 bp DNA.<sup>1</sup> This method has been widely applied for the quantitative analysis while in the presence of damaged DNA, the fluorescence intensity might be reduced due to electro-transfer quenching.<sup>2</sup> HeLa, 3T3-L1, and Raw264.7 cells were seeded on 24-well plates at  $5 \times 10^4$  cells/well and cultured for 24 h. The medium was replaced by Opti-MEM (500  $\mu$ L), into which P14/YOYO-1-DNA complexes were added at 1  $\mu$ g/mL. Arg9, TAT, PLR, and LPF2000 were used as internal controls. After incubation at 37 °C for 4 h, cells were washed with cold PBS containing heparin (20 IU/mL) for three times and subjected to flow cytometry analysis. Cells without any treatment served as the blank. The uptake level was expressed as the percentage of YOYO-1-DNA-positive cells.

siRNA was covalently labeled with FAM, and the uptake of P14/FAM-siRNA complexes in Raw 264.7 cells was evaluated in the same method as described above.

### **CLSM analysis**

The cellular internalization and endosomal escape of P14/YOYO-1DNA complexes were evaluated by CLSM observation and compared to Arg9/YOYO-1DNA complexes. Briefly, HeLa cells were seeded on coverslips in a 6-well plate at  $2 \times 10^4$  cells/well and cultured for 24 h. Complexes were added at 1  $\mu$ g DNA/well and the cells were cultured for 4 h at 37 °C. Following wash by PBS-heparin for three times, the nuclei were stained with DAPI and the endosomes/lysosomes were stained with LysoTracker<sup>®</sup> Red (Invitrogen). Cells were then observed by CLSM (Zeiss-700, Germany).

The cellular internalization and endosomal escape of P14/FAM-siRNA complexes were observed in the same method as described above and were compared to Arg9/FAM-siRNA complexes.

### ***In vitro* transfection**

HeLa, 3T3-L1, and Raw264.7 cells were seeded on 96-well plates at  $1 \times 10^4$  cells/well and incubated for 24 h prior to transfection studies. The medium was replaced by Opti-MEM, into which P14/DNA complexes at various N/P ratios were added at 0.1  $\mu$ g DNA/well. After incubation for 4 h, the complexes were removed and fresh media were added. Cells were further cultured for 20 h, following by determination of luciferase expression using a Bright-Glo Luciferase assay kit and cellular protein level using a BCA kit. Results were expressed as relative luminescence unit (RLU) associated with 1 mg of protein. Arg9/DNA complex, TAT/DNA complex, and PLR/DNA complex were also evaluated as controls. LPF2000 was used according to the manufacturer's protocol.

### ***In vitro* TNF- $\alpha$ knockdown**

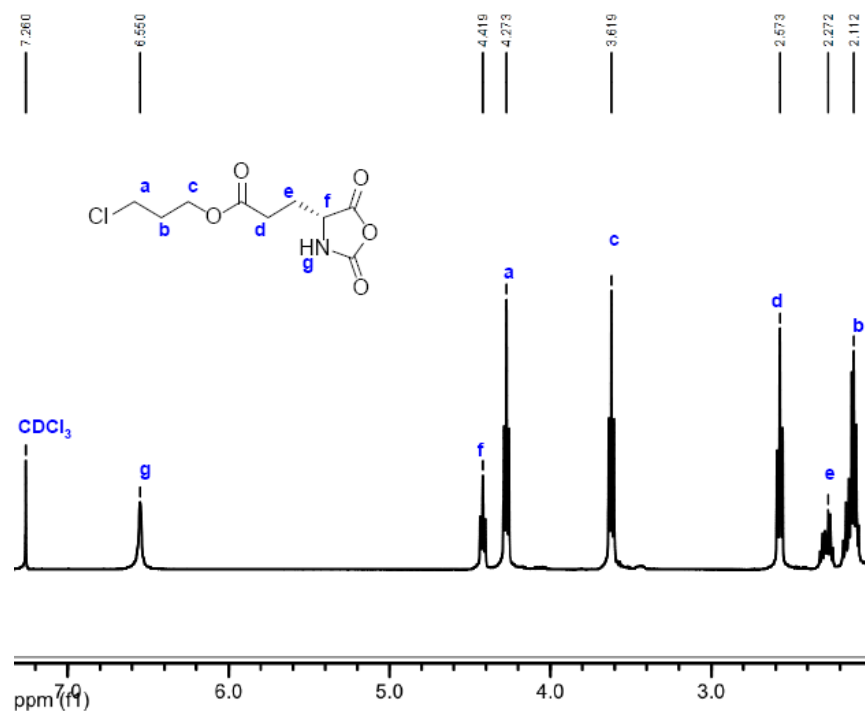
RAW 264.7 cells were seeded on 24-well plates at  $5 \times 10^4$  cells/well and cultured for 24 h. The medium was changed to Opti-MEM (500  $\mu\text{L}$ /well) and P14/siRNA complexes at various N/P ratios were added at 0.2  $\mu\text{g}$  siRNA/mL. Following incubation for 4 h, complexes were removed and fresh media were added. Cells were further cultured for 20 h before LPS stimulation (100  $\text{ng} \cdot \text{mL}^{-1}$ ) for 3 h. Extracellular TNF- $\alpha$  production was quantified by ELISA (R&D Systems, MN, USA), and the silencing efficiency was denoted as the percentage of TNF- $\alpha$  levels of the control cells which did not receive complexes treatment but were stimulated with LPS.

**Supplementary Table S1.** Sequence of TNF- $\alpha$  siRNA and Scrambled siRNA

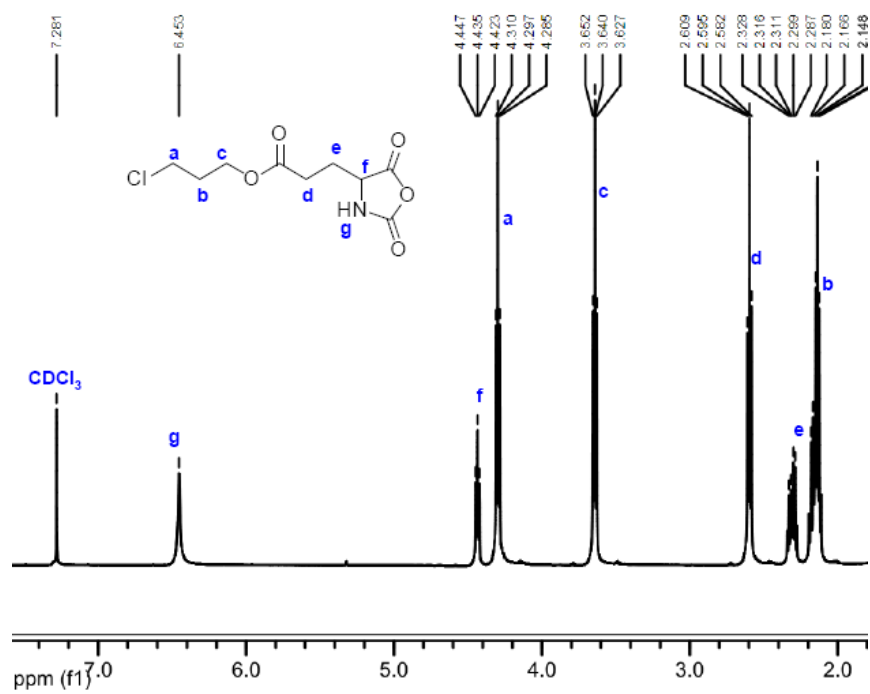
	Sequences
TNF- $\alpha$ sense	5'-UAACAAGCCAGAGUUGGUCdTdT-3'
TNF- $\alpha$ antisense	5'-GACCAACUCUGGCUUGUUAdTdT-3'
Scr sense	5'-UUCUCCGAACGUGUCACGUdTdT-3'
Scr antisense	5'-ACGUGACACGUUCGGAGAAAdTdT-3'



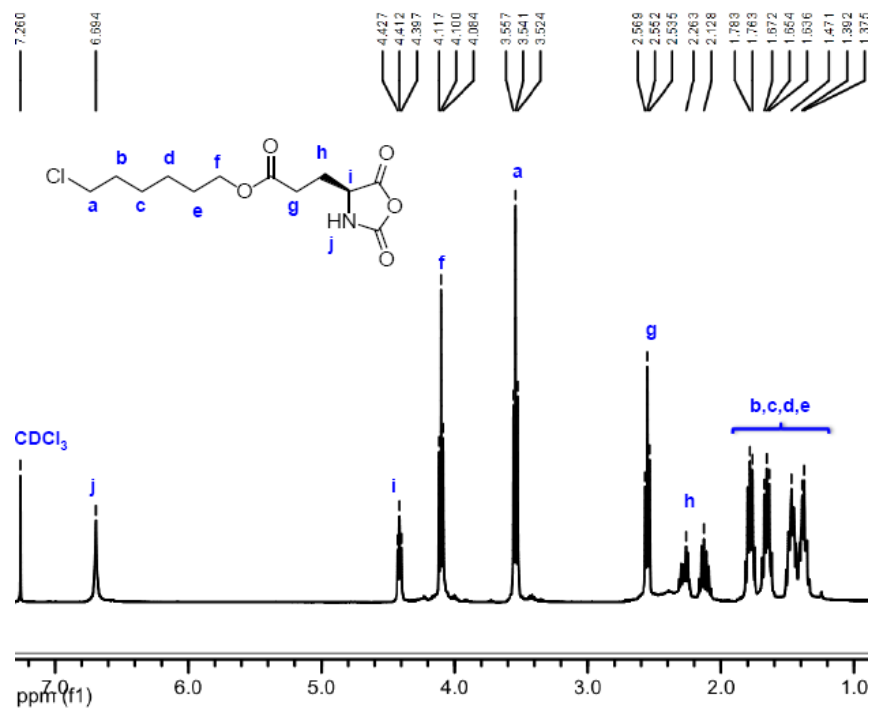
## Supplementary Figures



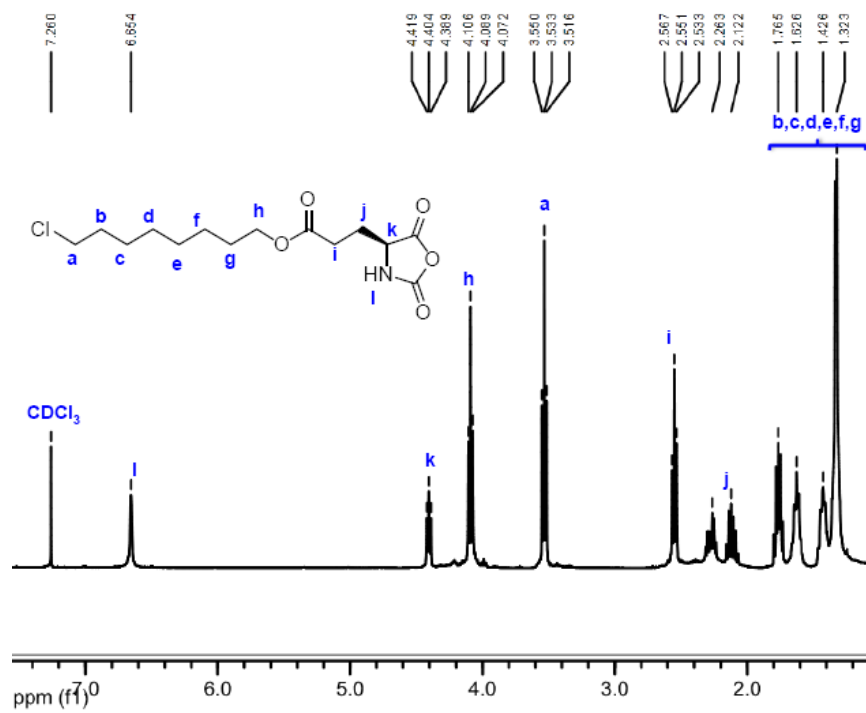
**Figure S1.** <sup>1</sup>H NMR spectrum of γ-chloropropyl-D-glutamic acid based N-carboxyanhydrides in CDCl<sub>3</sub>.



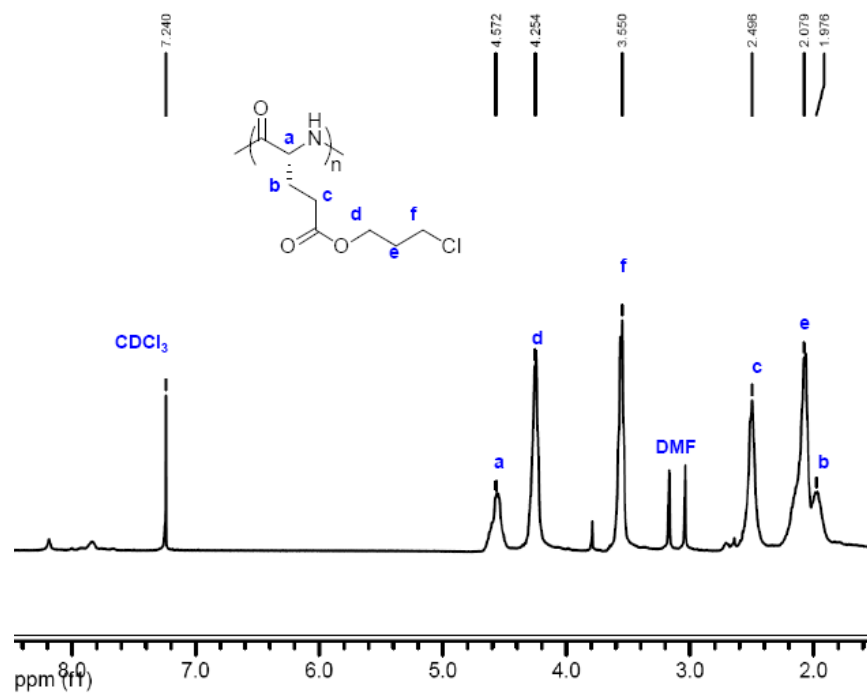
**Figure S2.**  $^1\text{H}$  NMR spectrum of  $\gamma$ -chloropropyl-DL-glutamic acid based *N*-carboxylanhydrides in  $\text{CDCl}_3$ .



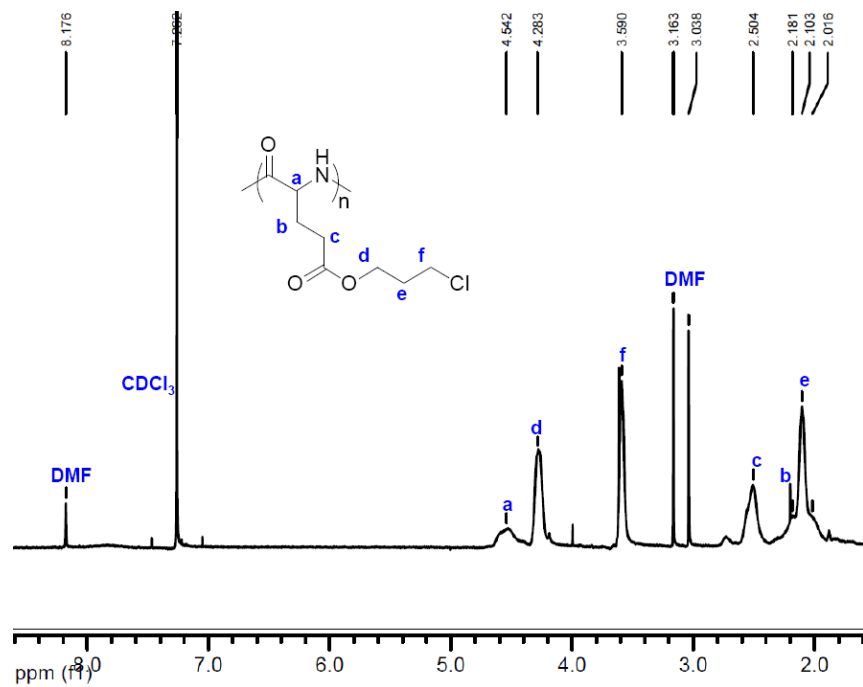
**Figure S3.**  $^1\text{H}$  NMR spectrum of  $\gamma$ -chlorohexyl-L-glutamic acid based *N*-carboxylanhydrides in CDCl<sub>3</sub>.



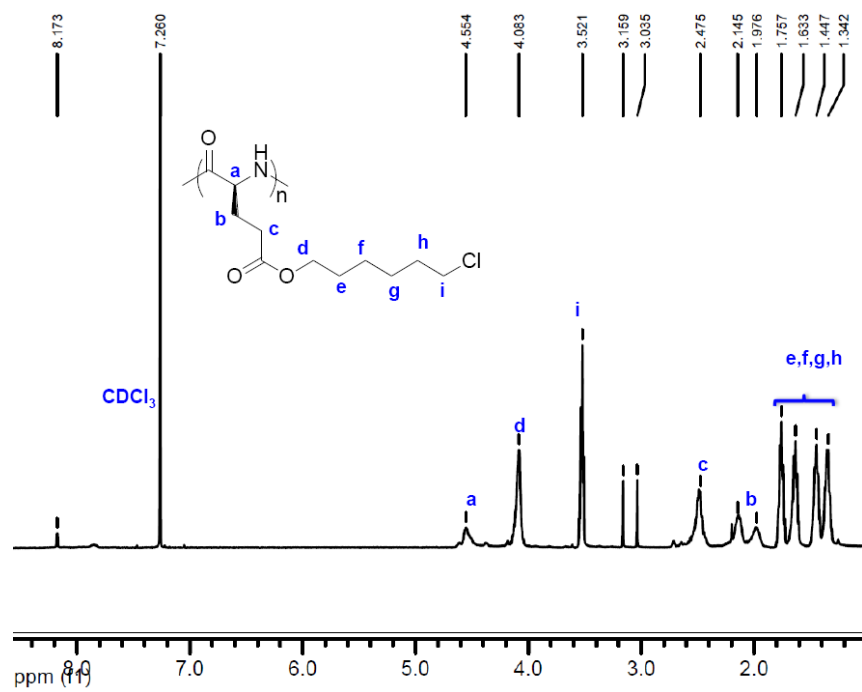
**Figure S4.**  $^1\text{H}$  NMR spectrum of  $\gamma$ -chlorooctyl-L-glutamic acid based *N*-carboxylanhydrides in  $\text{CDCl}_3$ .



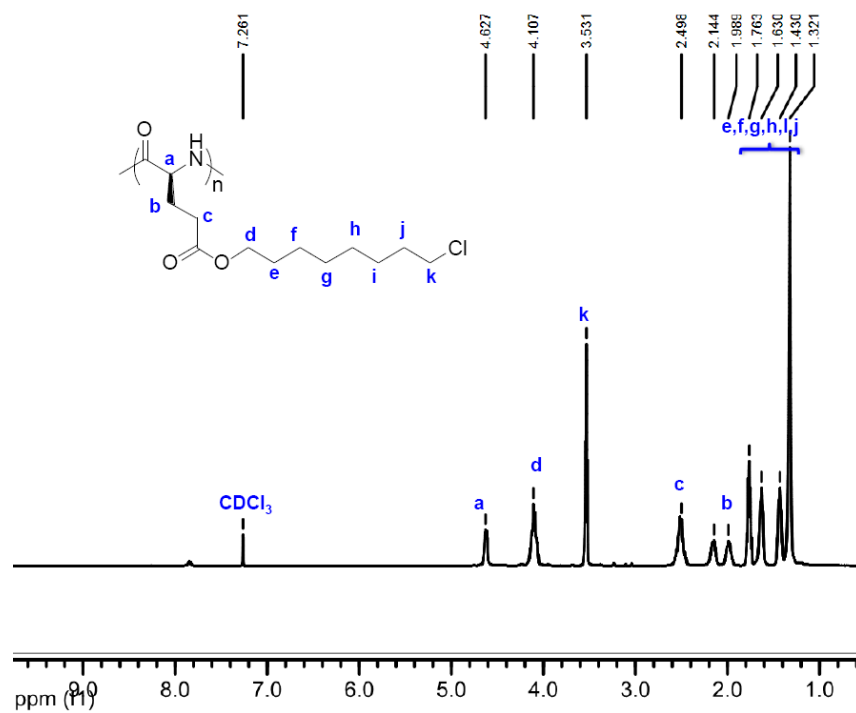
**Figure S5.** <sup>1</sup>H NMR spectrum of PCPDG in CDCl<sub>3</sub>/CF<sub>3</sub>CO<sub>2</sub>D (v/v=85/15).



**Figure S6.**  $^1\text{H}$  NMR spectrum of PCPDLG in  $\text{CDCl}_3/\text{CF}_3\text{CO}_2\text{D}$  (v/v=85/15).

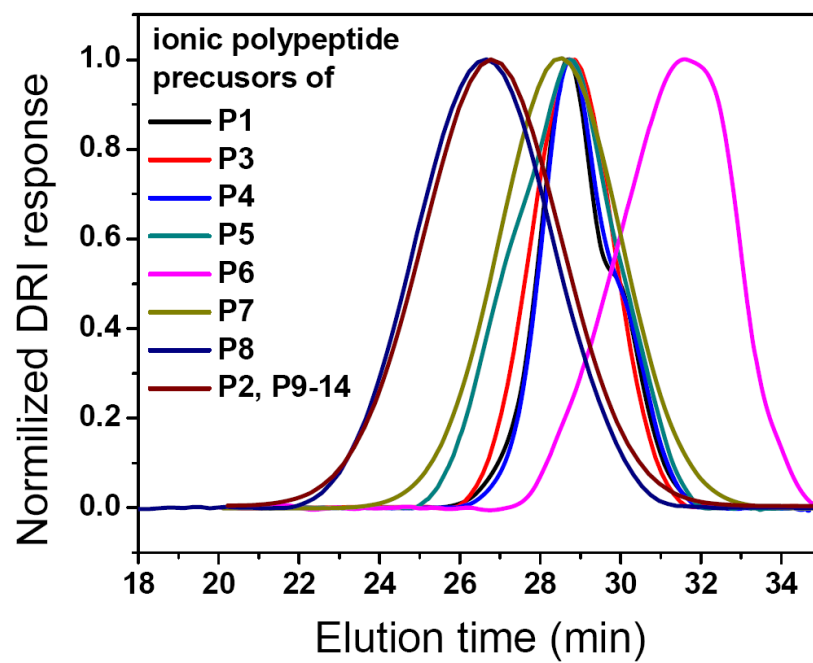


**Figure S7.** <sup>1</sup>H NMR spectrum of PCHLG in CDCl<sub>3</sub>/CF<sub>3</sub>CO<sub>2</sub>D (v/v=85/15).

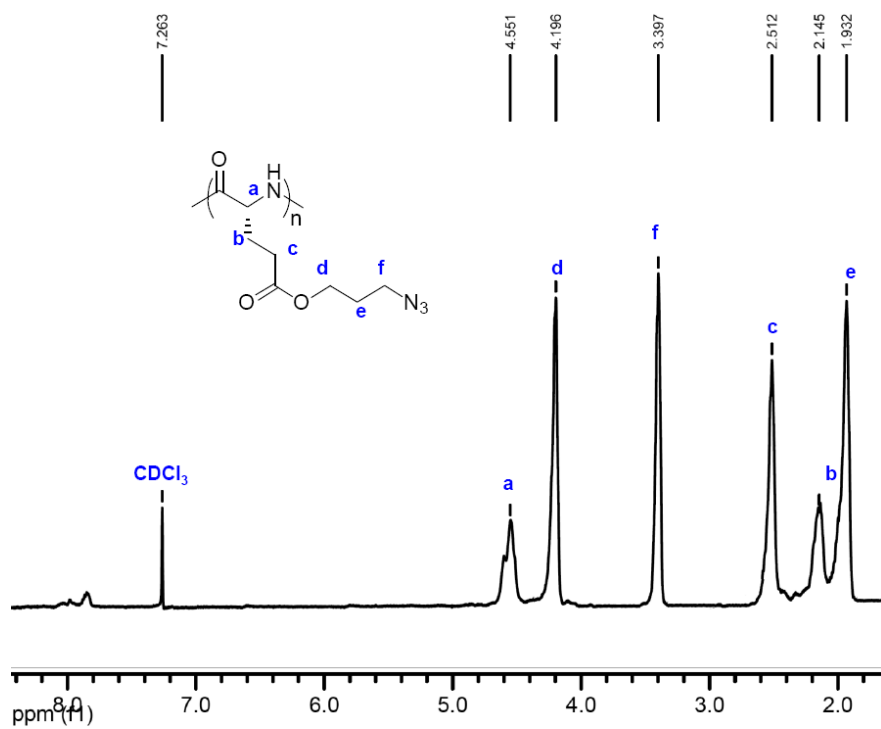


**Figure S8.** <sup>1</sup>H NMR spectrum of PCOLG in CDCl<sub>3</sub>/CF<sub>3</sub>CO<sub>2</sub>D (v/v=85/15).

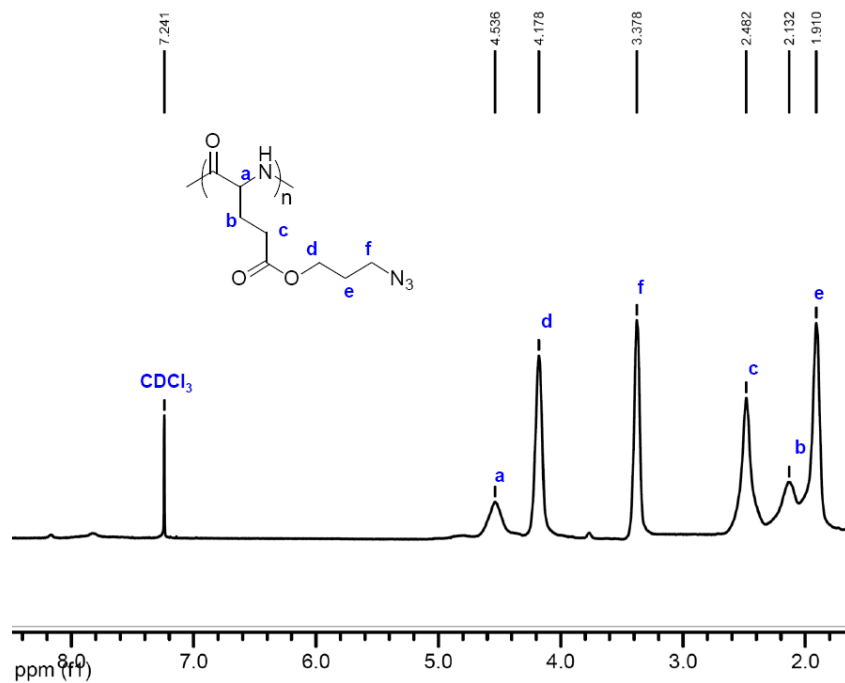




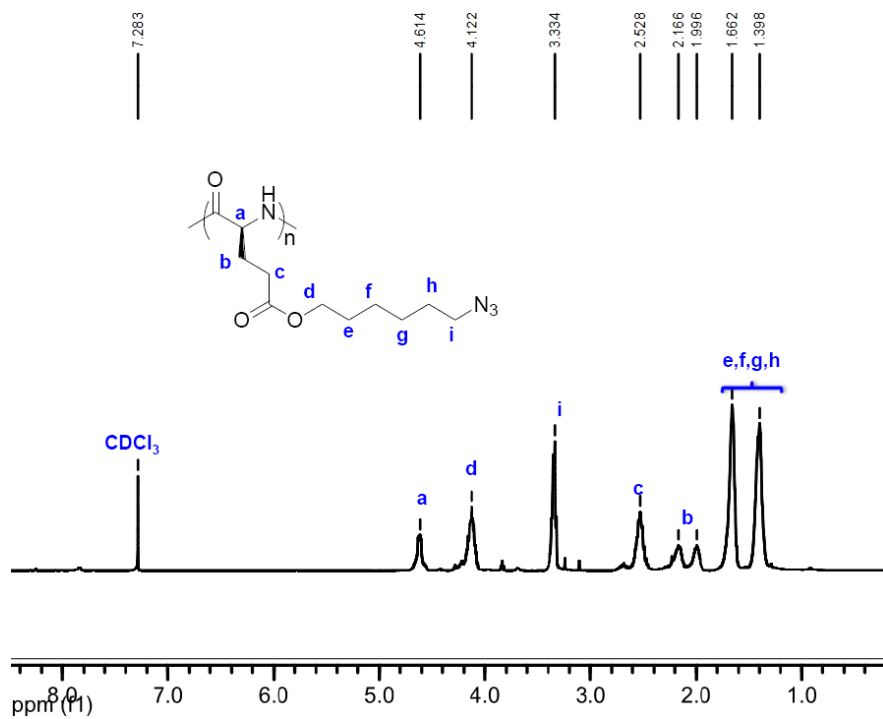
**Figure S9.** SEC traces of the precursors of guanidinium-rich polypeptides (P1-P14).



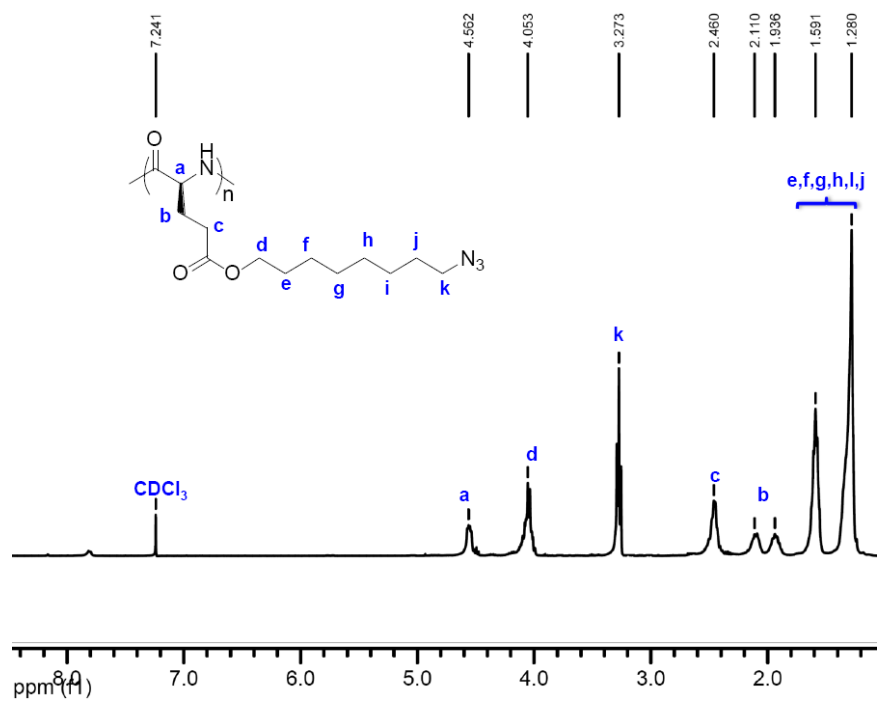
**Figure S10.** <sup>1</sup>H NMR spectrum of PAPDG in CDCl<sub>3</sub>/CF<sub>3</sub>CO<sub>2</sub>D (v/v=85/15).



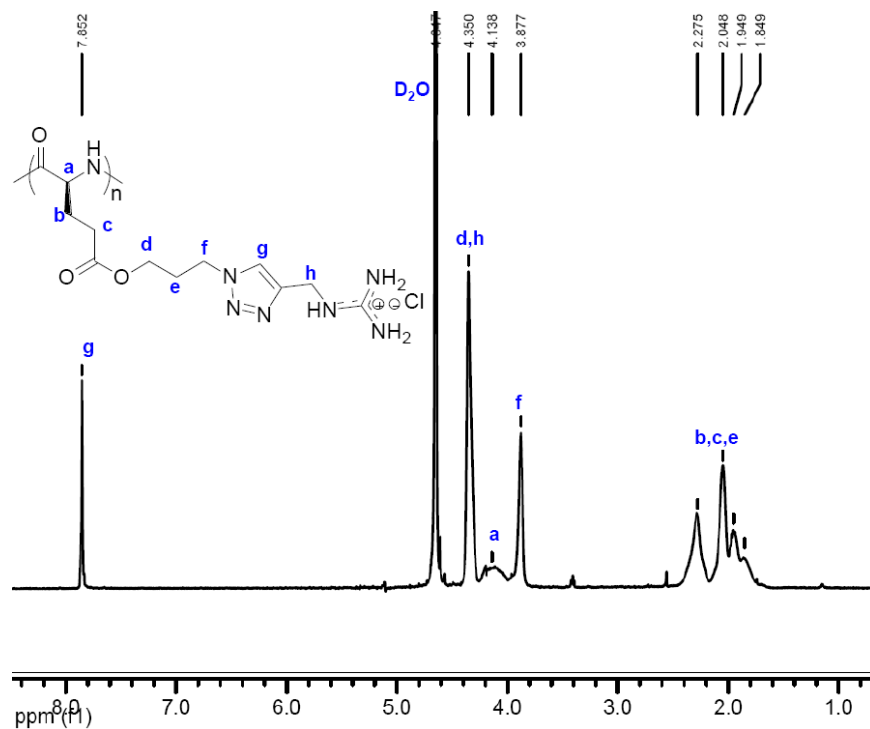
**Figure S11.**  $^1\text{H}$  NMR spectrum of PAPDLG in  $\text{CDCl}_3/\text{CF}_3\text{CO}_2\text{D}$  (v/v=85/15).



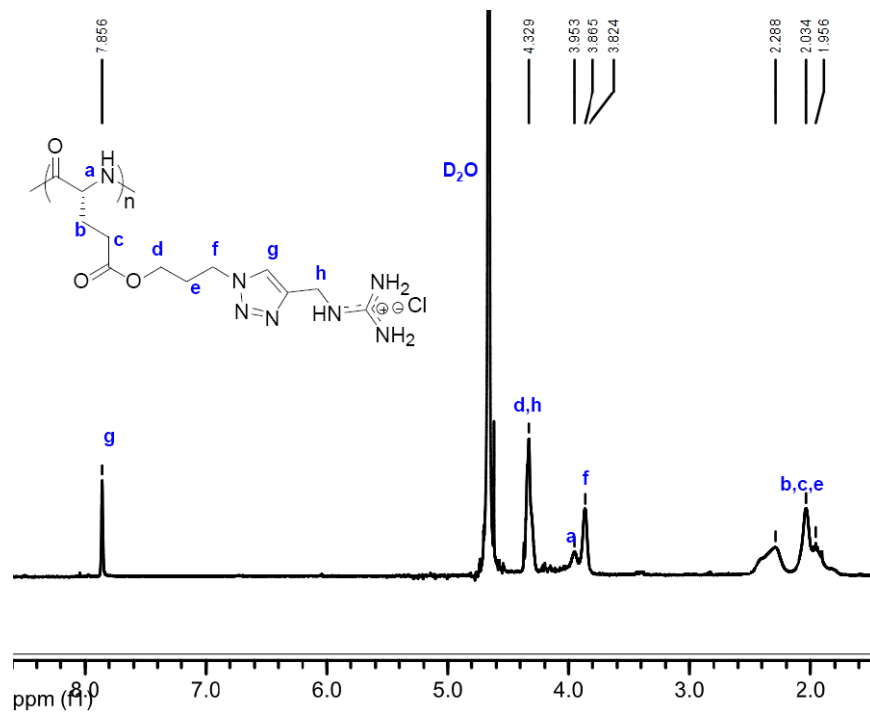
**Figure S12.** <sup>1</sup>H NMR spectrum of PAHLG in CDCl<sub>3</sub>/CF<sub>3</sub>CO<sub>2</sub>D (v/v=85/15).



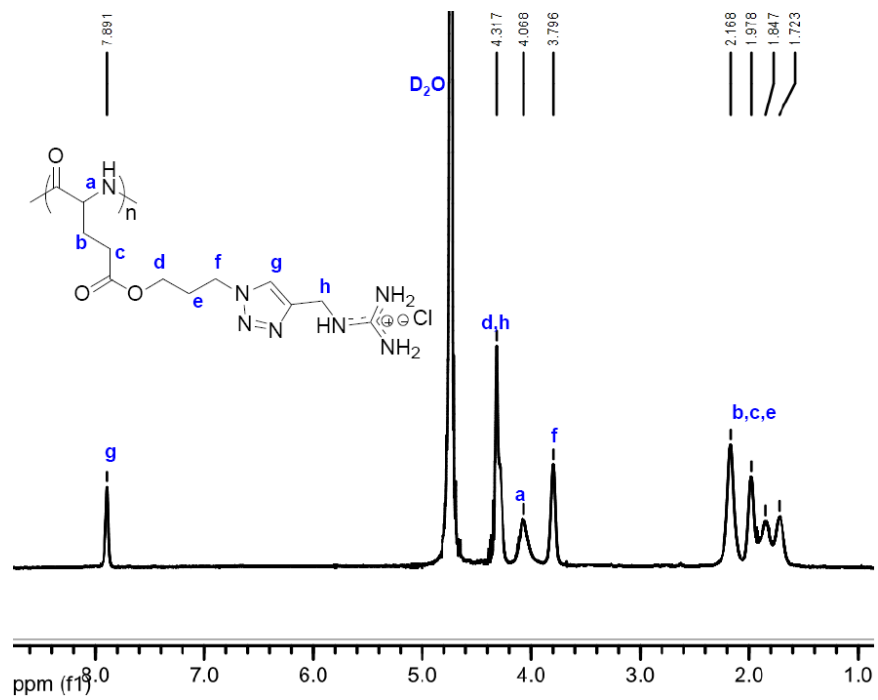
**Figure S13.**  $^1\text{H}$  NMR spectrum of PAOLG in  $\text{CDCl}_3/\text{CF}_3\text{CO}_2\text{D}$  (v/v=85/15).



**Figure S14.**  $^1\text{H}$  NMR spectrum of P1 in  $\text{D}_2\text{O}$ .

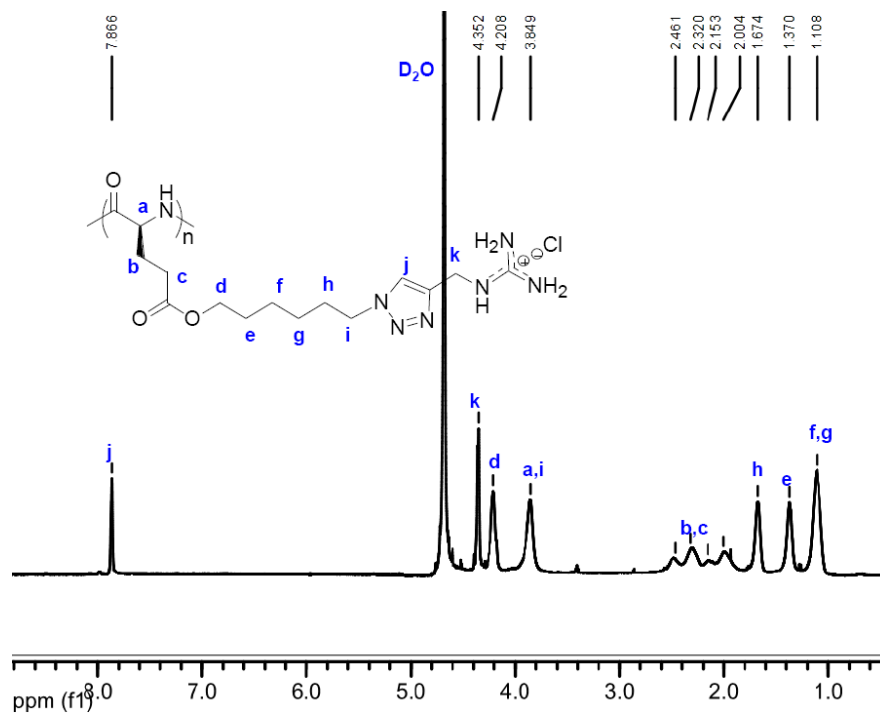


**Figure S15.** <sup>1</sup>H NMR spectrum of P2 in D<sub>2</sub>O.

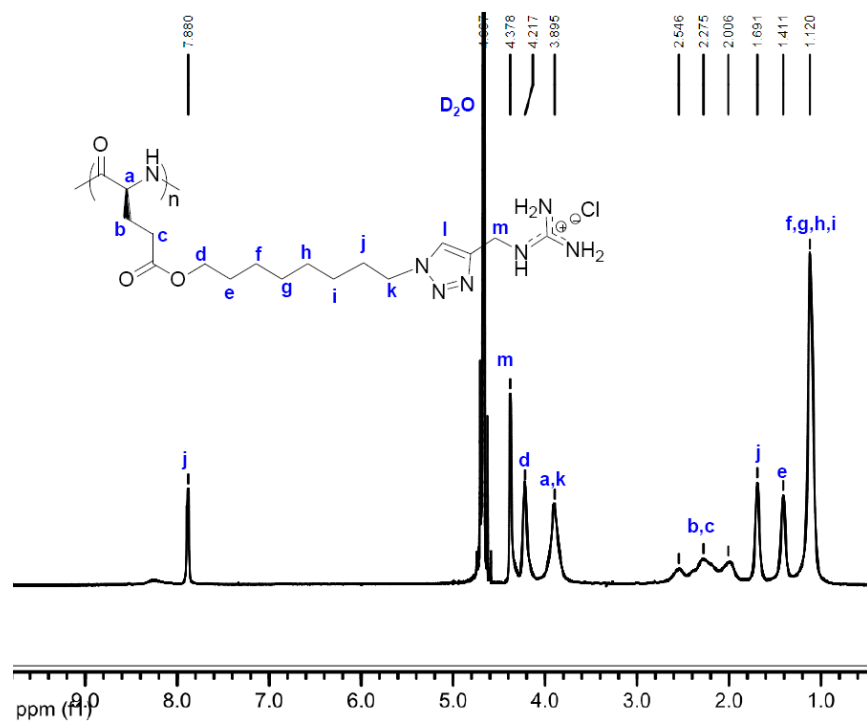


**Figure S16.** <sup>1</sup>H NMR spectrum of P3 in D<sub>2</sub>O.

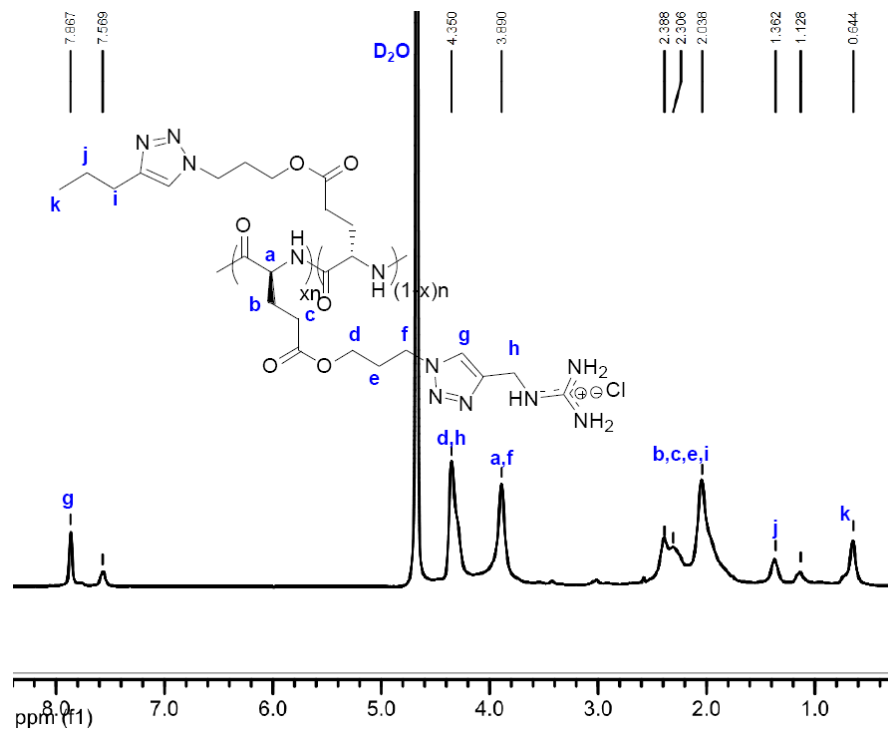




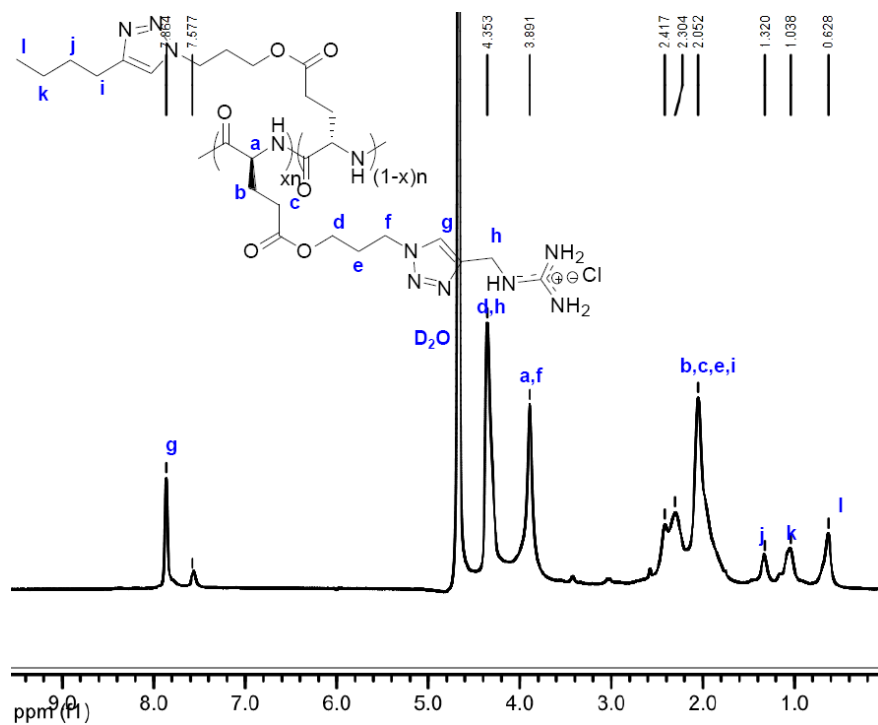
**Figure S17.**  $^1\text{H}$  NMR spectrum of P5 in  $\text{D}_2\text{O}$ .



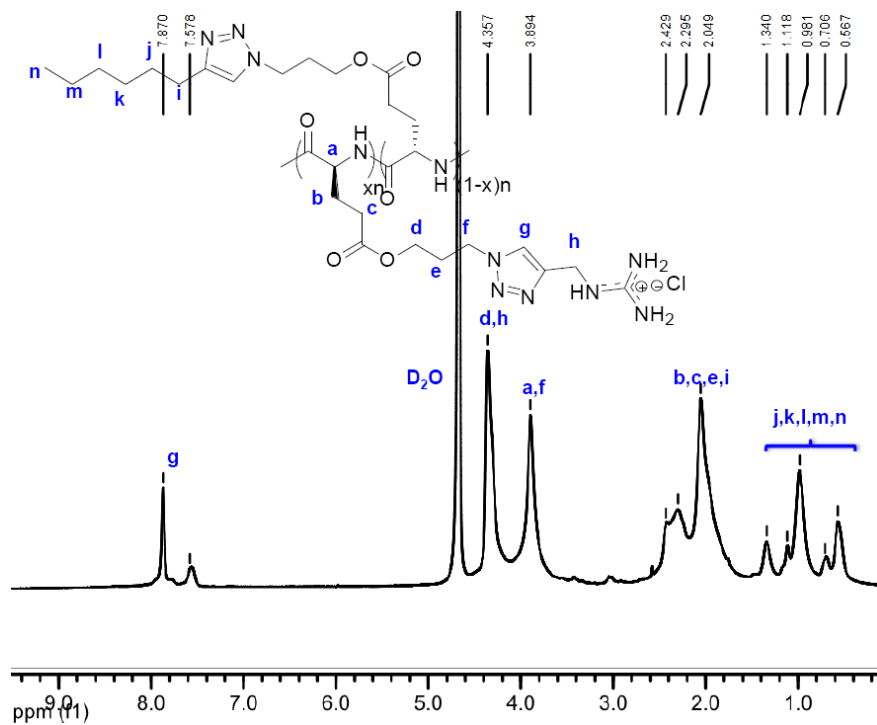
**Figure S18.**  $^1\text{H}$  NMR spectrum of P6 in  $\text{D}_2\text{O}$ .



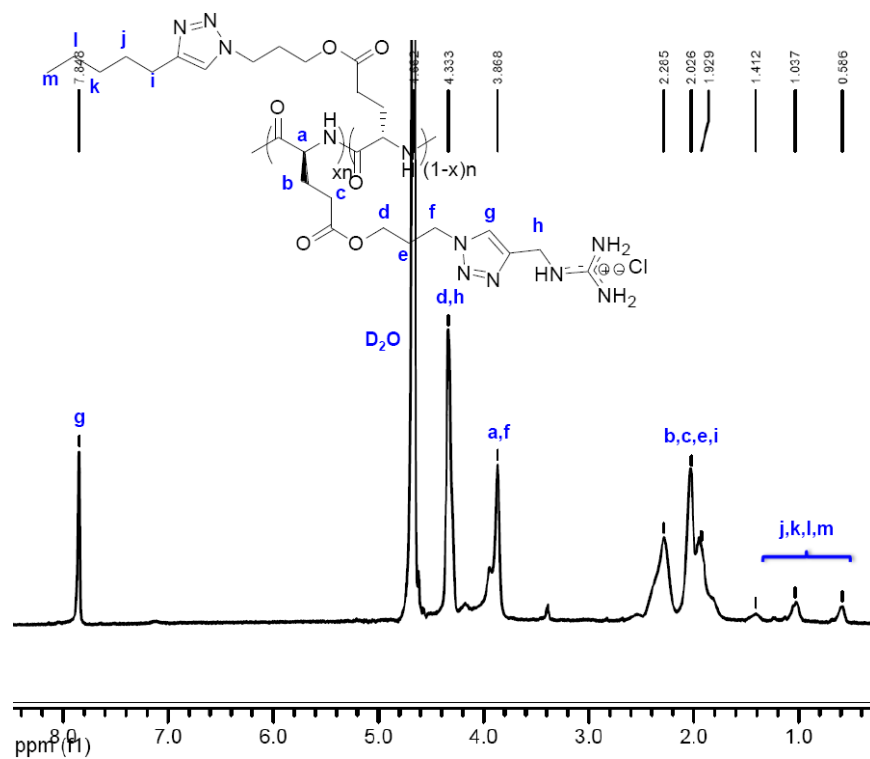
**Figure S19.**  $^1\text{H}$  NMR spectrum of P9 in  $\text{D}_2\text{O}$ .



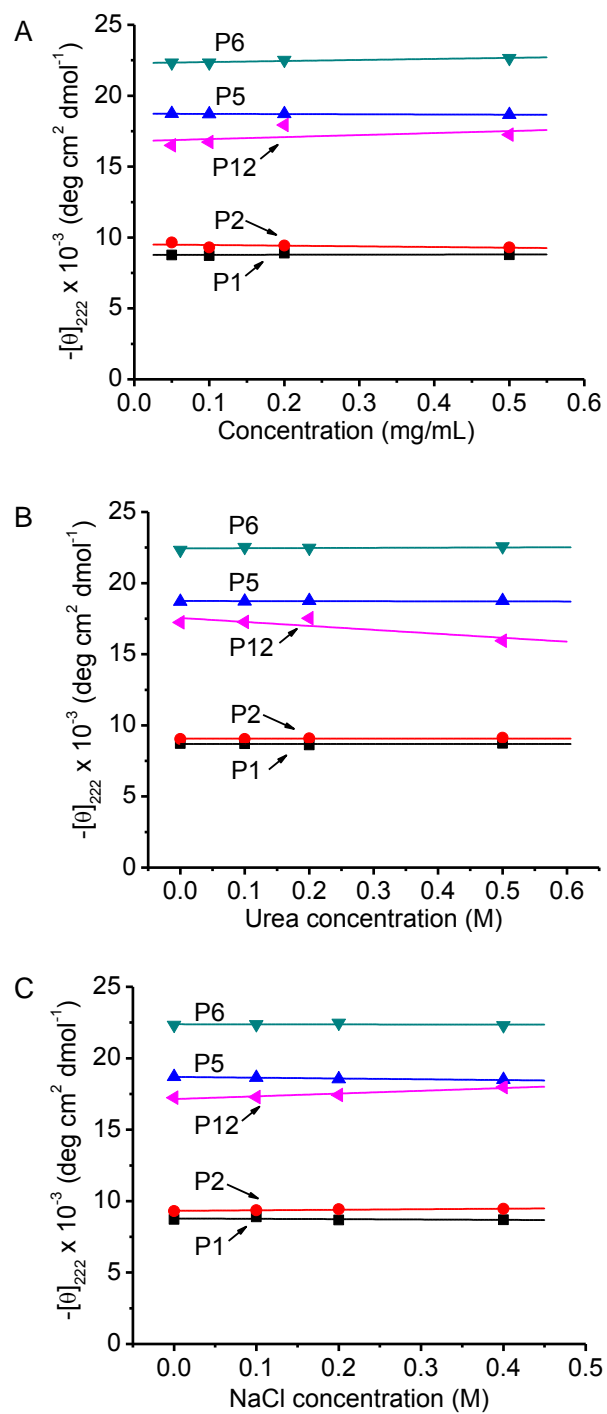
**Figure S20.** <sup>1</sup>H NMR spectrum of P10 in D<sub>2</sub>O.



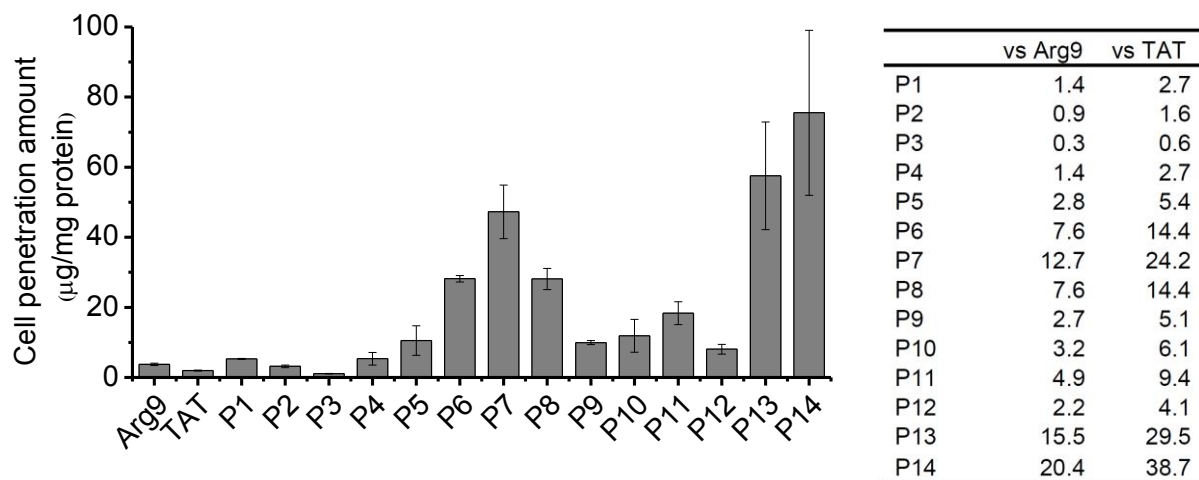
**Figure S21.**  $^1\text{H}$  NMR spectrum of P12 in  $\text{D}_2\text{O}$ .



**Figure S22.** <sup>1</sup>H NMR spectrum of P13 in D<sub>2</sub>O.

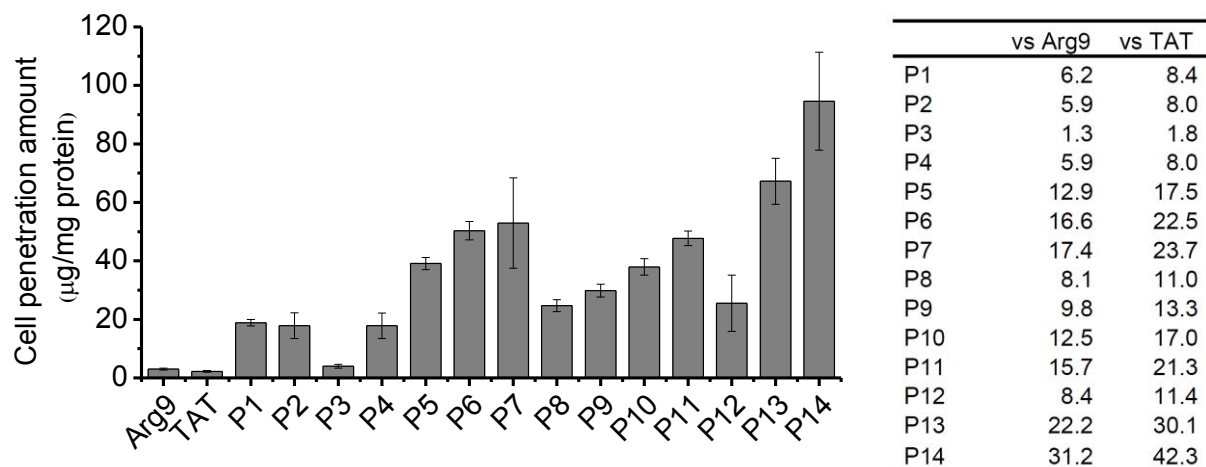


**Figure S23.** Molar ellipticity of guanidinium-rich polypeptides at 222 nm as a function of the polymer concentration (A), urea concentration (B), and salt concentration (C).

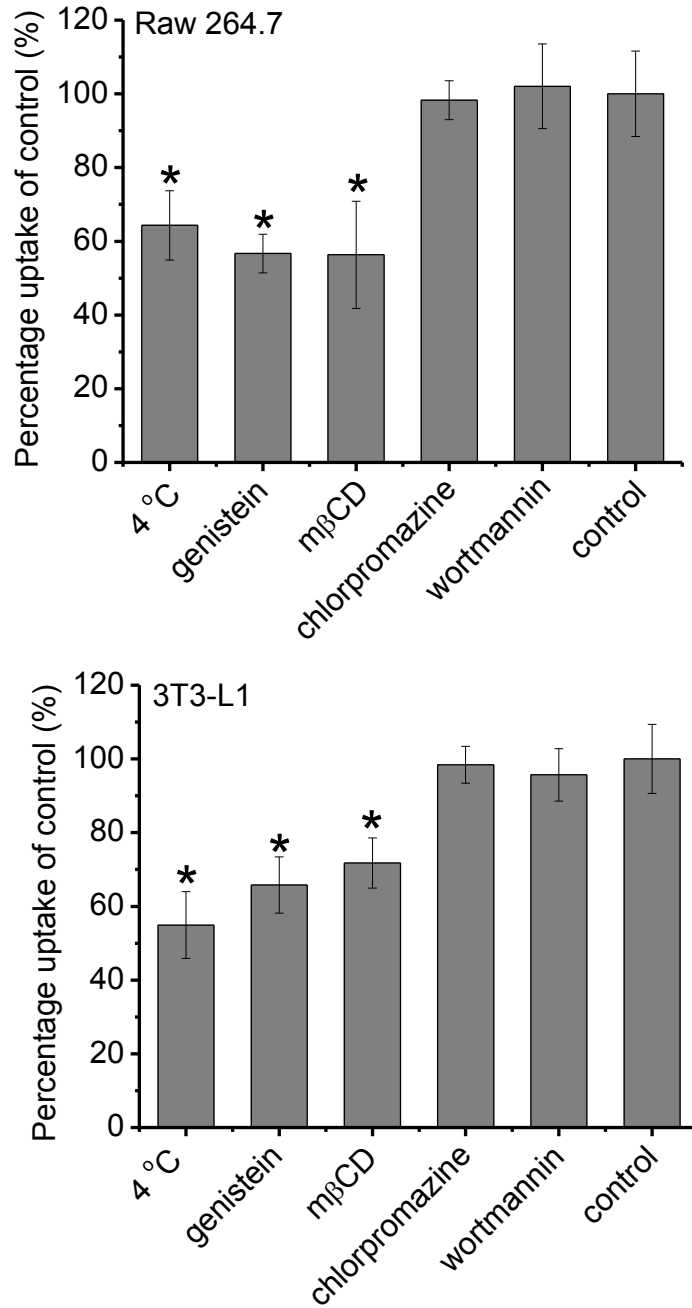


**Figure S24.** Cell uptake levels of rhodamine-labeled polypeptides in Raw 264.7 cells following incubation for 2 h at 37 °C. Results were expressed as  $\mu\text{g}$  polypeptide associated with 1 mg cellular protein (n=3).

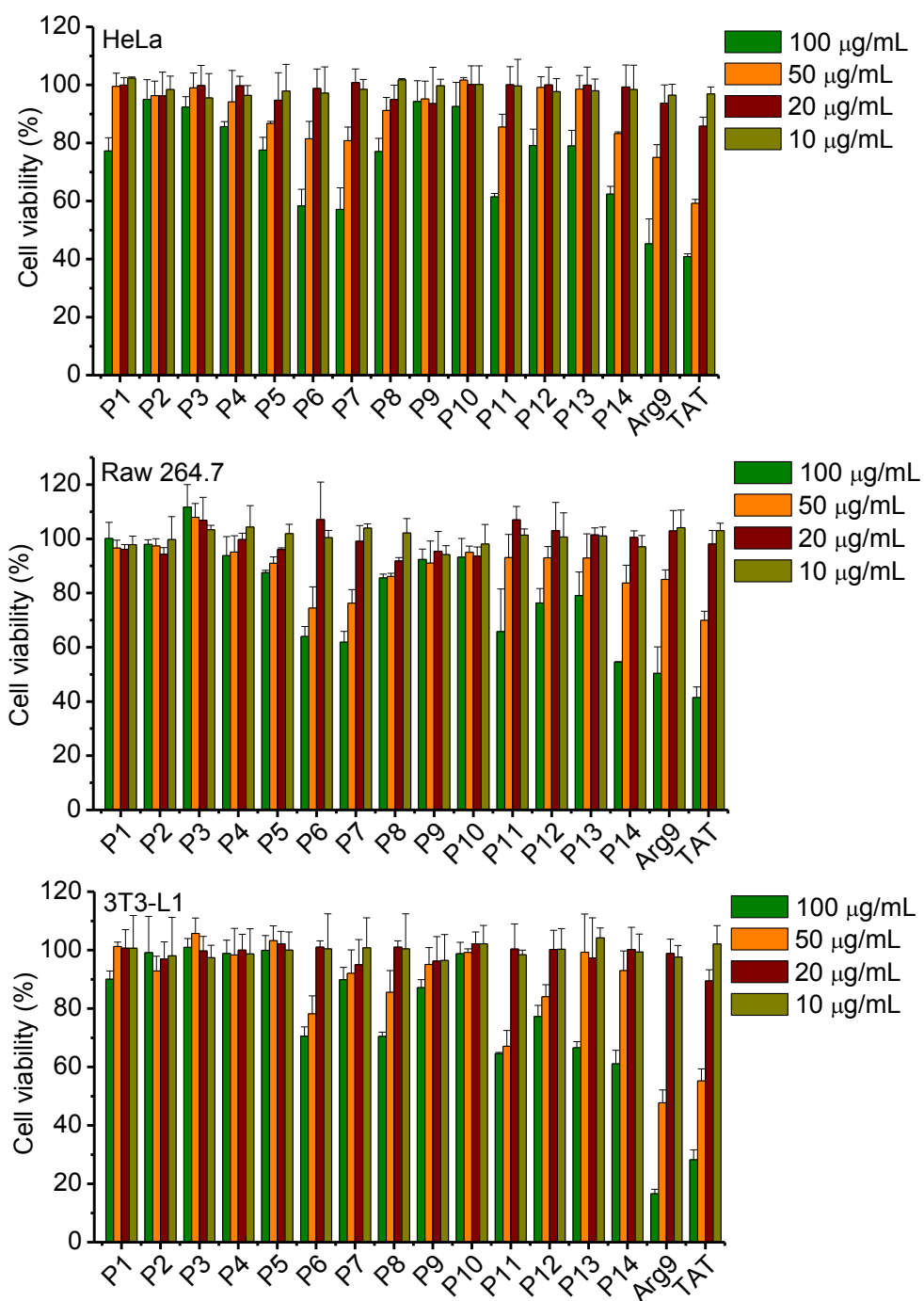




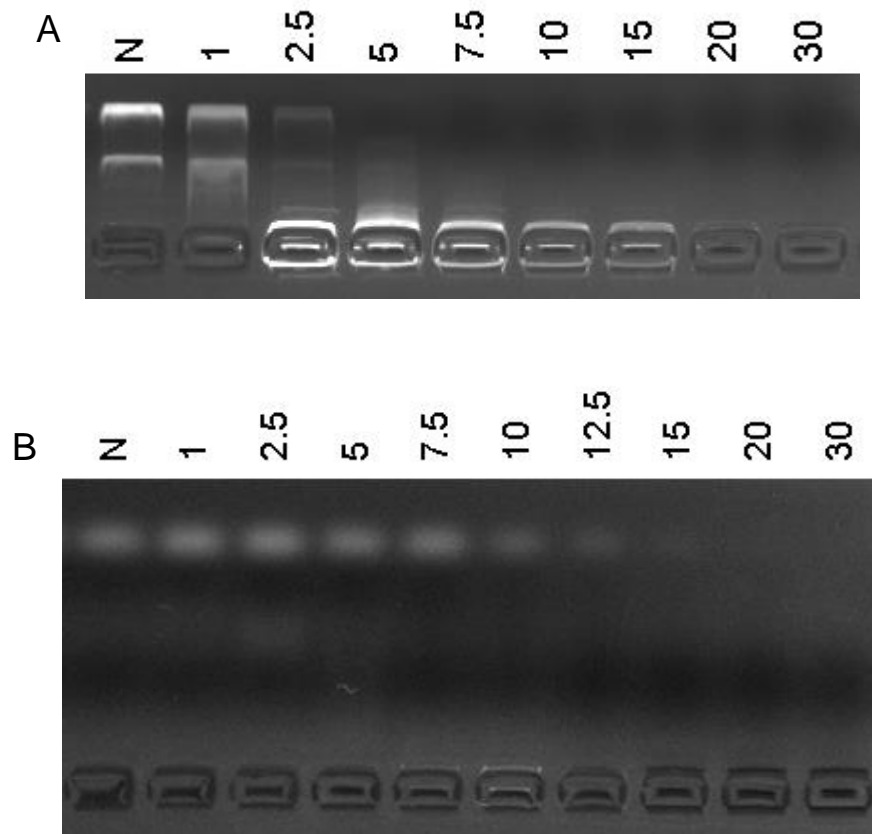
**Figure S25.** Cell uptake levels of rhodamine-labeled polypeptides in 3T3-L1 cells following incubation for 2 h at 37 °C. Results were expressed as  $\mu\text{g}$  polypeptide associated with 1 mg cellular protein (n=3).



**Figure S26.** Internalization levels of RhB-P14 in Raw 264.7 and 3T3-L1 cells in the presence of various endocytic inhibitors (n=3). Results were expressed as percentage of control cells without inhibitor treatment.

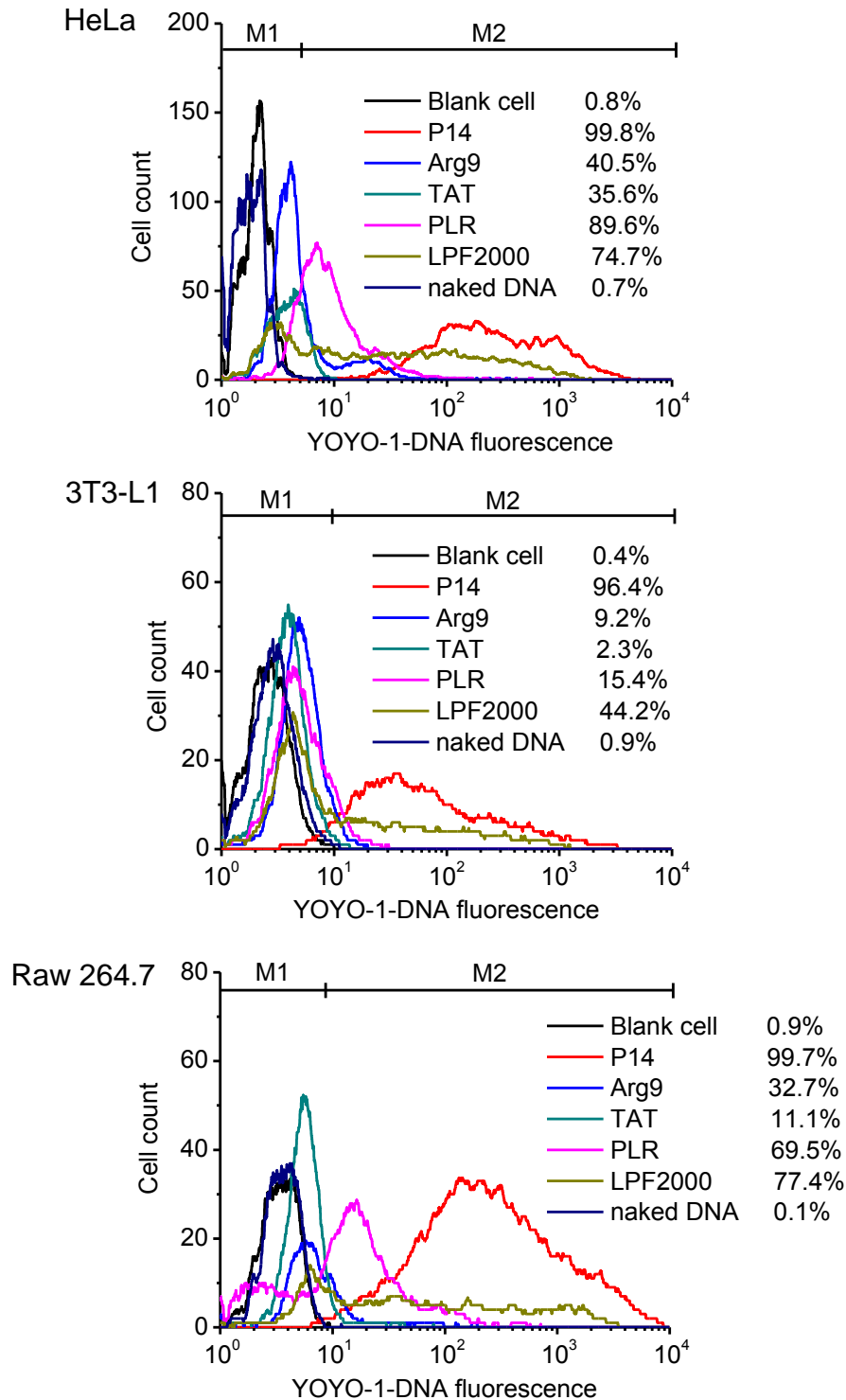


**Figure S27.** Viability of HeLa, Raw 264.7, and 3T3-L1 cells following treatment with polypeptides for 4 h as determined by the MTT assay (n=3).

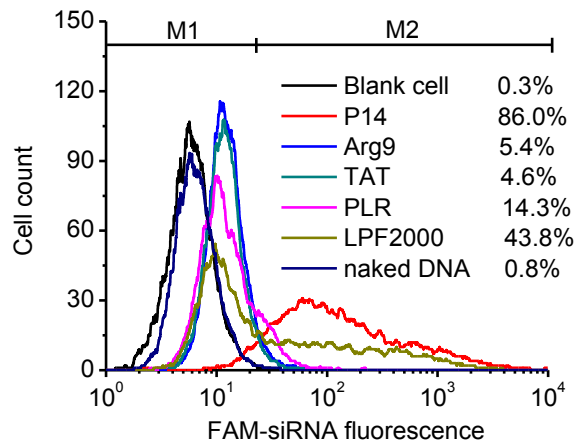


**Figure S28.** Gel retardation assay showing the condensation of DNA (A) and siRNA (B) by P14 at various N/P ratios. N represents naked DNA or siRNA.

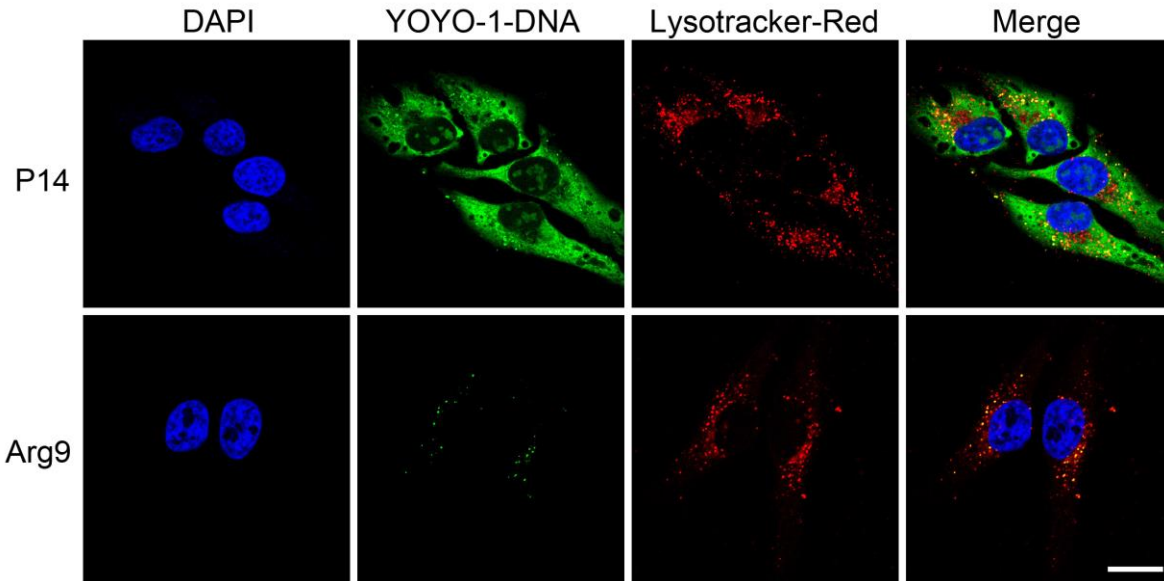
The cationic P14 was able to condense the anionic DNA and siRNA molecules at the N/P ratios higher than 5 and higher than 15, respectively.



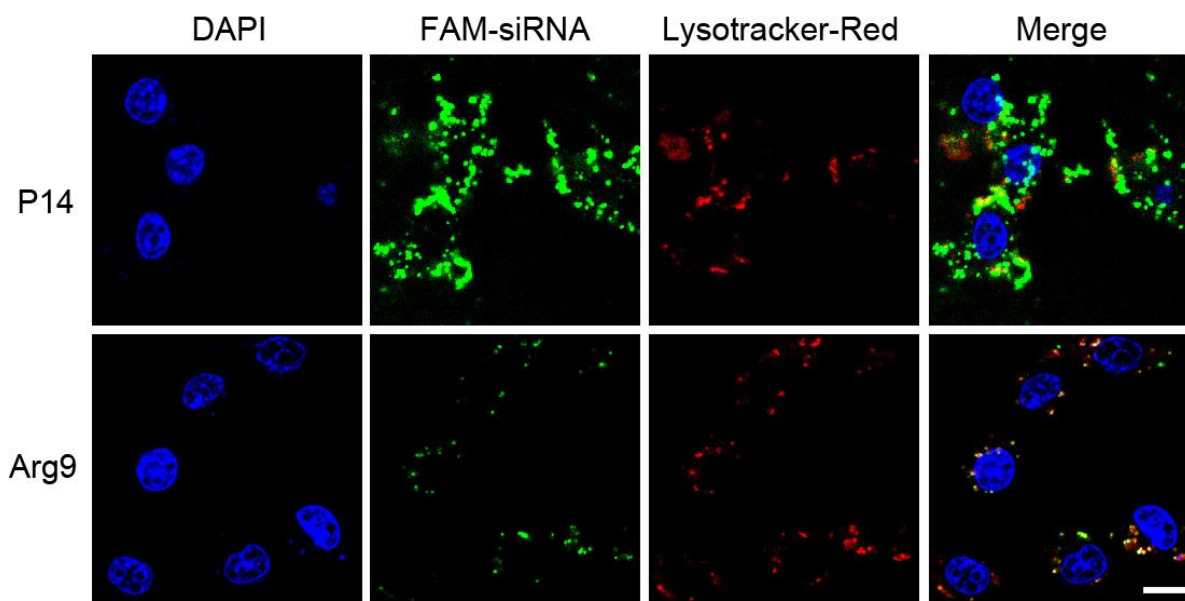
**Figure S29.** Flow cytometry analysis on the cell uptake of P14/YOYO-1-DNA complexes (N/P ratio = 15) in HeLa, 3T3-L1, and Raw 264.7 cells. Arg9, TAT, PLR, and LPF2000 at the N/P ratio of 15, 15, 10, and 5 were used controls, respectively. M1 represents YOYO-1-DNA negative cells and M2 represents YOYO-1-DNA positive cells. The percentage of M2 cells was listed.



**Figure S30.** Flow cytometry analysis on the cell uptake of P14/FAM-siRNA (N/P ratio = 30) complexes in Raw 264.7 cells. Arg9, TAT, PLR, and LPF2000 at the N/P ratio of 20, 20, 15, and 7.5, respectively. M1 represents FAM-siRNA negative cells and M2 represents FAM-siRNA positive cells. The percentage of M2 cells was listed.

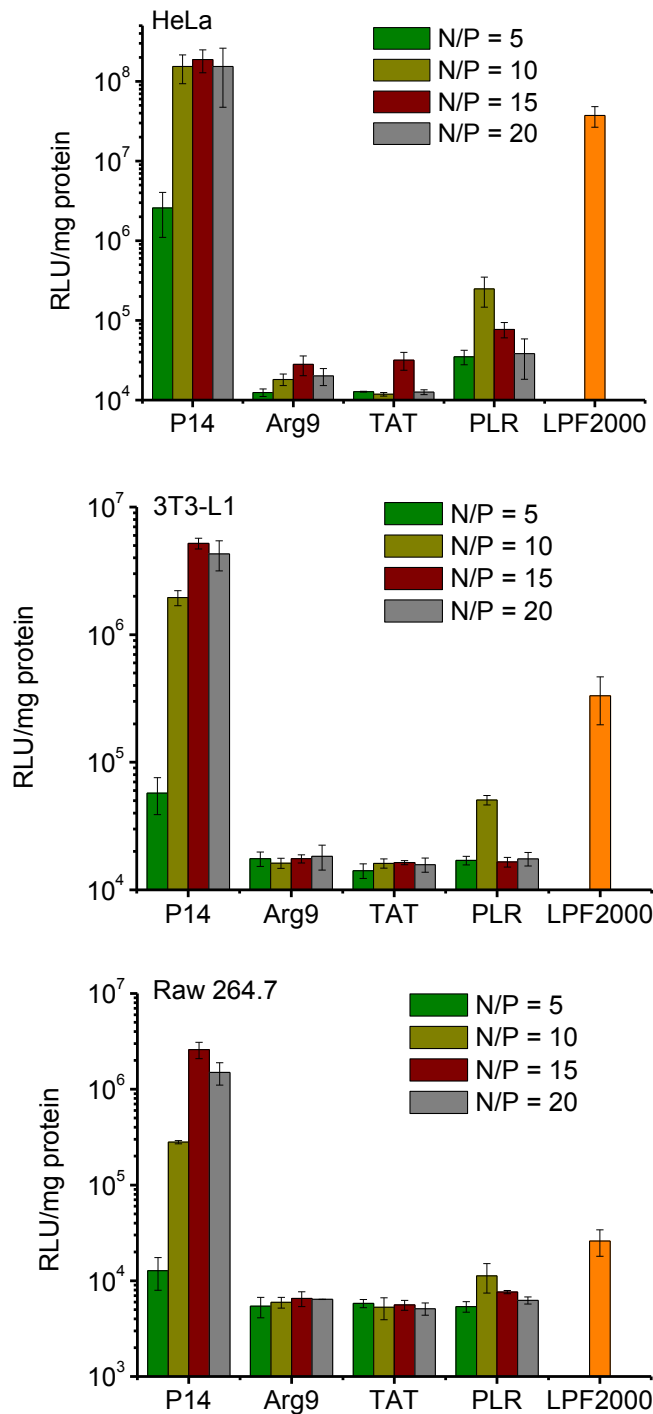


**Figure S31.** CLSM images of HeLa cells following 4-h treatment with P14/YOYO-1-DNA (N/P = 15) complexes or Arg9/YOYO-1-DNA complexes (N/P = 15). The endosomal/lysosomal compartments were stained with Lysotracker Red, while the nuclei were stained with DAPI. Bar = 20  $\mu$ m.

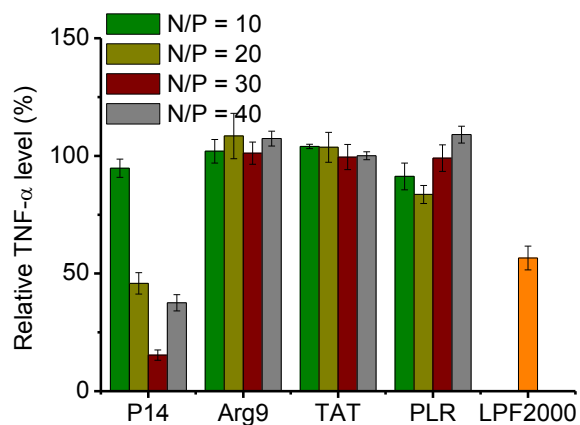


**Figure S32.** CLSM images of Raw 264.7 cells following 4-h treatment with P14/FAM-siRNA (N/P = 30) complexes or Arg9/FAM-siRNA complexes (N/P = 20). The endosomal/lysosomal compartments were stained with Lysotracker Red, while the nuclei were stained with DAPI. Bar = 10  $\mu$ m.





**Figure S33.** Transfection efficiencies of P14/DNA, Arg9/DNA, TAT/DNA, and PLR/DNA complexes in HeLa, 3T3-L1, and Raw 264.7 cells at various N/P ratios. LPF2000 as the commercial transfect reagent was used as a control at the optimal N/P ratio of 5 accordingly to the manufacturer's protocol.



**Figure S34.** Silencing efficiencies of P14/TNF- $\alpha$  siRNA, Arg9/TNF- $\alpha$  siRNA, TAT/TNF- $\alpha$  siRNA, and PLR/TNF- $\alpha$  siRNA complexes in Raw 264.7 cells at various N/P ratios. LPF2000 as the commercial transfect reagent was used as a control at the optimal N/P ratio of 7.5 accordingly to the manufacturer's protocol.

#### Reference

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