Guanidine-Containing Methacrylamide (Co)polymers *via* aRAFT: Toward a Cell Penetrating Peptide Mimic

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Experimental

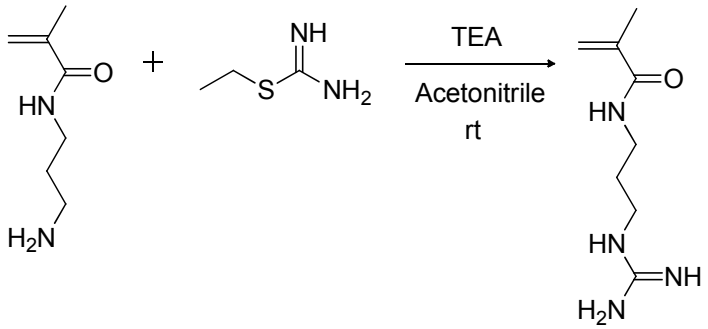
Materials and Methods:

All chemicals were purchased from Aldrich at the highest available purity and used as received unless otherwise noted. 4,4´-Azobis(4-cyanopentanoic acid) (V-501, a gift from Wako Pure Chemicals Industries, Ltd.) was recrystallized from methanol. 4-Cyano-4-(ethylsulfanylthiocarbonylsulfanyl)pentanoic acid (CEP) was synthesized according to literature procedure.[1] N-(3-Aminopropyl)methacrylamide hydrochloride was bought from Polysciences, Inc. and used as received. HPMA was synthesized according to literature procedure.(ref) Polymerizations were conducted using standard Schlenk conditions. Deionized water (DI H_2O) was obtained from a Barnstead NANO-Pure reverse osmosis/filtration unit (resistivity of 18.0 MΩ). ^1H and ^{13}C NMR was obtained on a Bruker 300 MHz. Polymers synthesized in this study were analyzed by aqueous size exclusion chromatography (ASEC) using an eluent of 1 wt% acetic acid in HPLC H_2O/0.10 M LiBr at a flow rate of 0.25 mL/min at 25˚ C. Eprogen Inc. CATSEC columns (100, 300, and 1000 Å) were used along with a Polymer Laboratories LC1200 UV/vis detector, Wyatt Optilab DSP interferometric refractometer (λ = 690 nm), and a Wyatt DAWN-DSP multiangle laser light scattering (MALLS) detector (λ = 633 nm). Monomer conversions were determined by comparing the integral of the vinyl peaks and the 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) peak at 0 δ using a Bruker 300 MHz NMR. The d*n*/d*c* of PHPMA and PGPMA were determined in the above eluent using a Bausch and Lomb refractometer to be 0.170 and 0.180, respectively. The absolute molecular weights and polydispersity indices were calculated using Wyatt ASTRA SEC/LS software package.

Synthesis of 3-guanidinopropyl methacrylamide (GPMA):

3 -guanidinopropyl methacrylamide (GPMA) was synthesized according to a modified literature procedure.[2] A 250-mL erlenmeyer flask was charged with N-(3-aminopropyl) methacrylamide hydrochloride (5 g, 27.98 mmol) and 20 mL of DI H_2O. The pH was adjusted by the addition of 50 wt% NaOH in water until the solution reached pH 11. The de-acidified N-(3-aminopropyl) methacrylamide hydrochloride solution was placed in a 250-mL seperatory funnel and the solution was washed 3 times with 100-mL of CH_2Cl_2 (300 mL), each time collecting the organic fraction. The CH_2Cl_2 was removed in vaccuo, leaving a transparent yellow oil. Full recovery of the product was assumed (4.0 g, 27.98 mmol) and added dropwise to a 100-mL round-bottomed flask charged with 2-ethyl-2-thiopseudourea hydrobromide (4.56g, 24.6 mmol), triethylamine (2.49g, 24.6 mmol), 20 mL of acetonitrile, and a teflon-coated stir bar. 1 mL of DI H_2O was added to the solution to fully dissolve the mixture. The reaction was set to stir at room

temperature for 16 hours. The reaction was stopped and the solvent was removed in vaccuo resulting in a viscous clear oil. The product was purified via column chromatography using 50:50 EtOH/EtOAc as the mobile phase over silica (Mesh size 40-60) (Rf = 0.4). The product was obtained as a clear oil, 3.3 g (72%). ^{1}H NMR (300 MHz, D_2O): δ (ppm) 1.68 (m, 2H), 1.77 (s, 3H), 3.07 (t, 2H), 3.18 (t, 2H), 5.29 (s, 1H), 5.54 (s, 1H). ^{13}C NMR (54 MHz, D_2O): δ (ppm) 17.6 (CH_3), 27.5 (CH_2), 36.5 (CH_2-N), 38.6 (CH_2-N), 121.0 (C), 138.9 (CH_2=C), 156.6 (C), 171.8 (C=O).



Scheme S1. Synthesis of GPMA

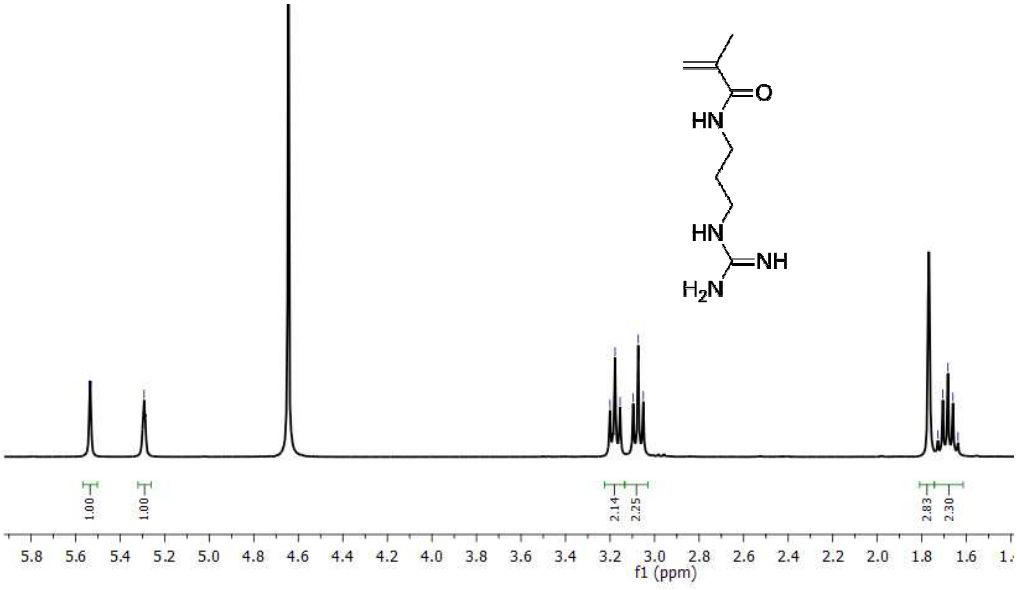


Figure S1. ^{1}H NMR confirming the structure of GPMA.

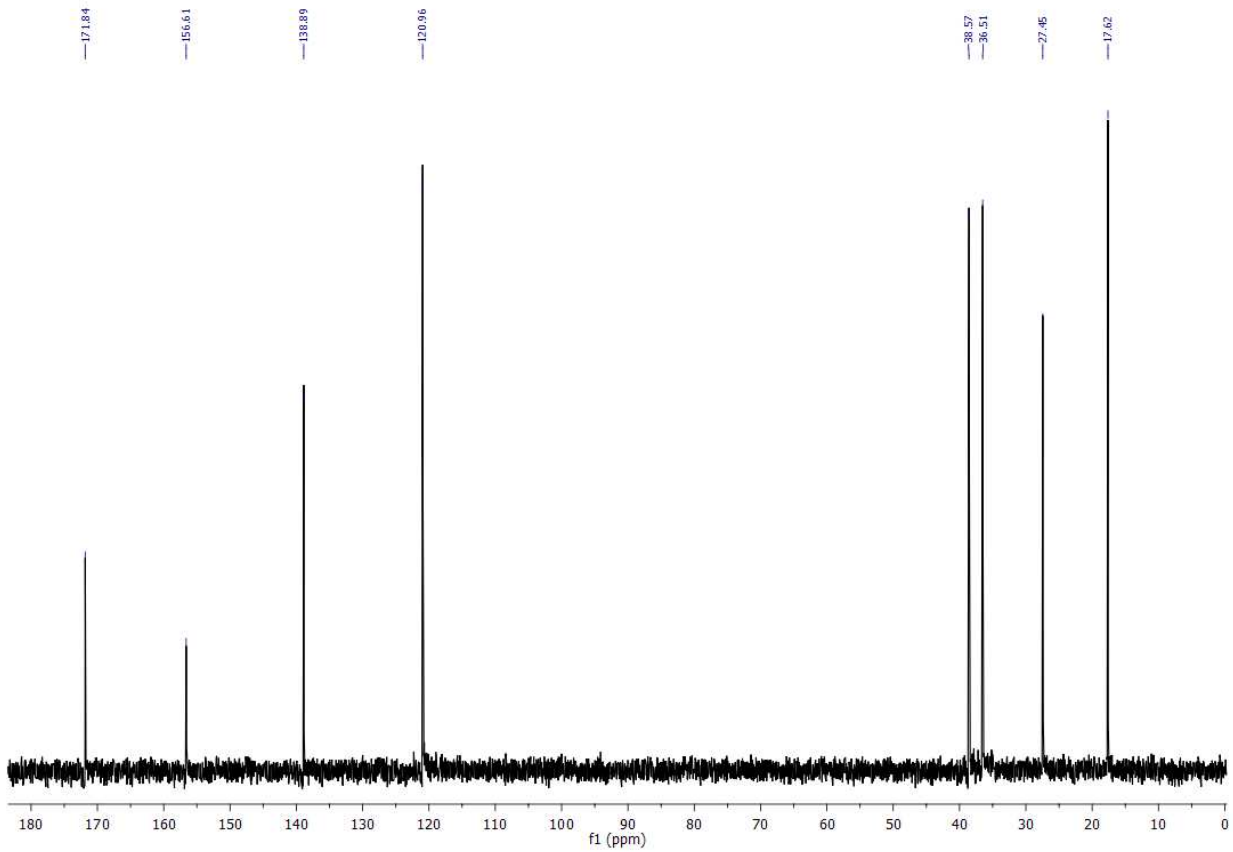


Figure S2. ^{13}C NMR confirming the structure of GPMA.

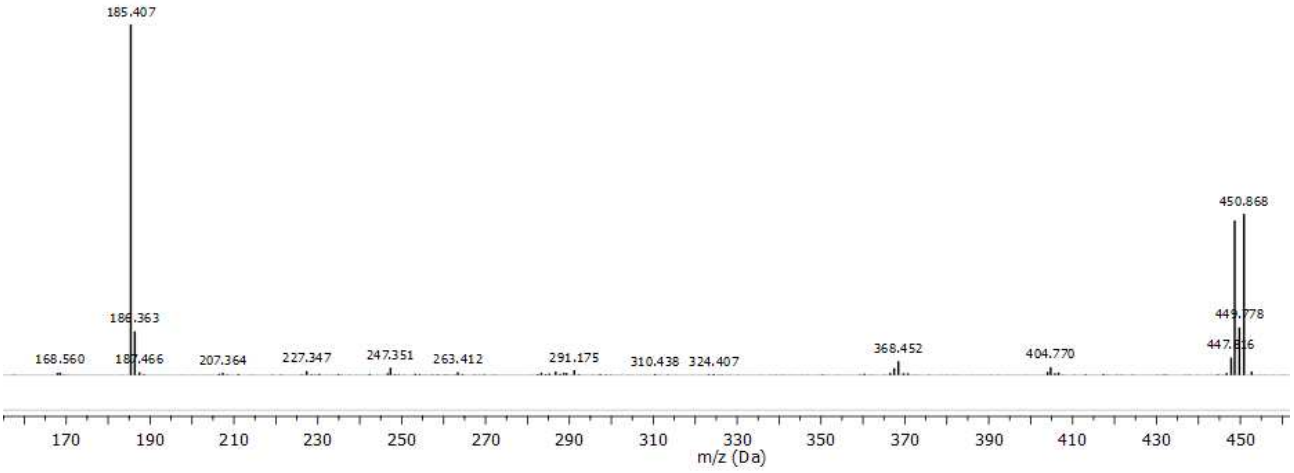


Figure S3. Mass spectroscopy showing the monomer at 185.407 Da

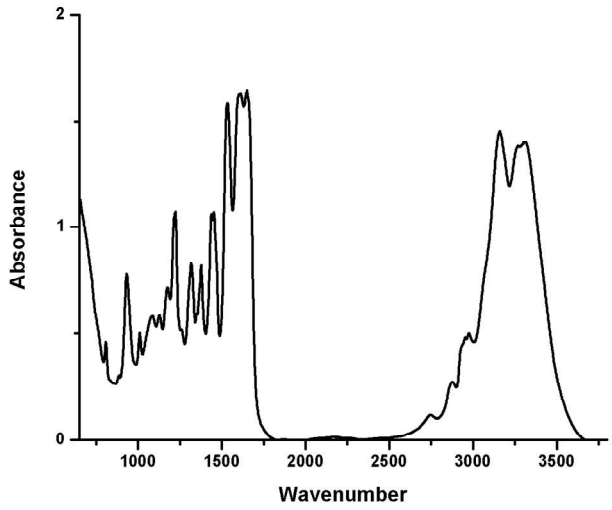
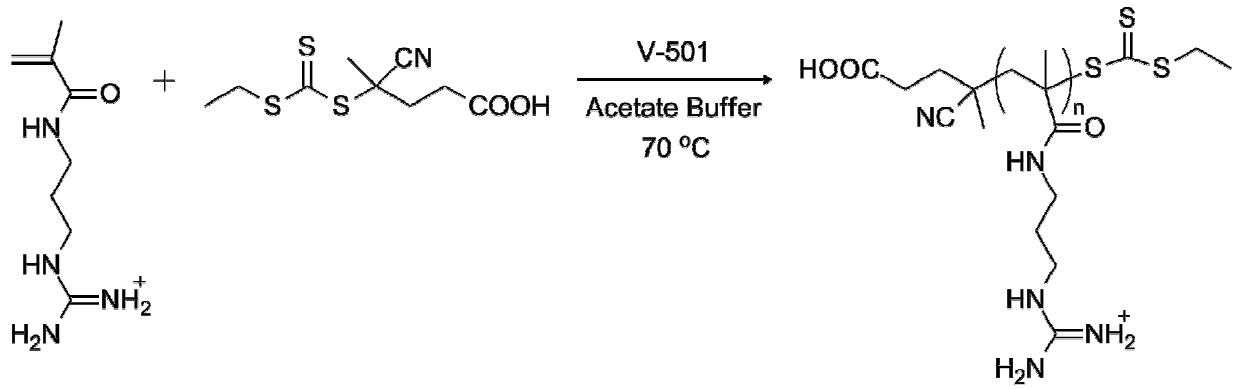


Figure S4. Attenuated Total Reflectance Infrared spectroscopy confirming monomer structure.

Synthesis of GPMA Macro CTA:

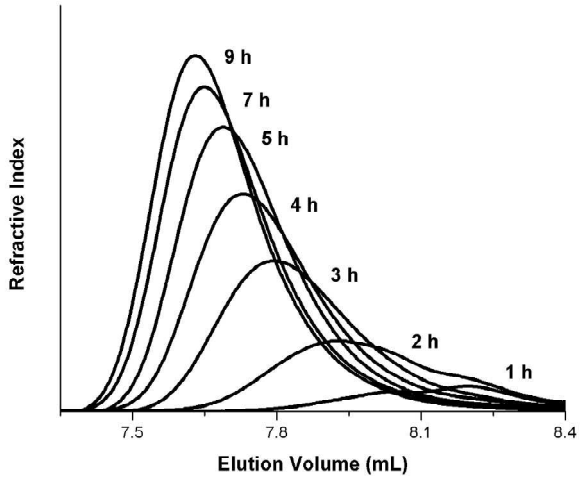
GPMA macroCTA was synthesized using V-501 as the primary radical source, CEP as the chain transfer agent, and aqueous acetic buffer (pH 5.2, 0.27 M acetic acid and 0.73 M sodium acetate) as the solvent at 70 ˚C. Briefly, GPMA (1.2 g, 6.49 mmol) was put into a 50 mL round-bottomed flask combined with 9 mL of acetic buffer. CEP (15.6 mg, 0.0594 mmol) and V-501 (3.3 mg, 0.0119 mmol) were dissolved in 1 mL of methanol before adding to the GPMA in order to avoid solubility issues with CEP. The initial monomer concentration was $[M]_0$= 0.65 M. GPMA homopolymer was prepared using a $[M]_0$:[CTA] ratio of 110/1, while the $[CTA]_0/[I]_0$ was 5/1. The round-bottomed flask was septum-sealed and purged with nitrogen for 40 minutes prior to overnight polymerization (19 h). The homopolymer was dialyzed against acidic (pH 3-4) DI water for 3 days at 4 ˚C, and subsequently dried by lyophilization. The resultant $GPMA_{78}$ (M_w/M_n = 1.08) was characterized using aqueous size exclusion chromatography (SEC) and ^1H NMR.



Scheme S2. Polymerization of GPMA using CEP as chain transfer agent and V-501 as the free radical initiator.

Polymerization Kinetics:

Polymerization kinetics were conducted using similar conditions to the GPMA macroCTA polymerization. Briefly, a 50 mL round-bottomed flask was charged with GPMA (0.95 g, 5.11 mmol) and 9 mL of HPLC H_2O at pH=5.5. CEP (24.7 mg, 0.0937 mmol) and V-501 (5.25mg, 0.0187 mmol) were dissolved in 1 mL of methanol before addition to the flask. The initial monomer concentration was $[M]_0$= 0.5 M and the $[M]_0$:[CTA] ratio was 55/1. The septum-sealed round-bottomed flask was purged for 1 h at room temperature prior to placing the flask in an oil bath at 70 ˚C. Aliquots were taken at pre-determined intervals using degassed syringes. Each aliquot was briefly exposed to the atmosphere and allowed to cool to room temperature to stop polymerization. Samples were characterized using aqueous SEC and ^{1}H NMR. Kinetics were also conducted at $[M]_0$ = 1.0 M using a $[M]_0$:[CTA] ratio of 150/1. Briefly, acetic buffer (pH = 5.2, 0.27 mol/L acetic acid and 0.73 mol/L sodium acetate)., GPMA (2.32 g, 12.54 mmol), CEP (22.0 mg, 0.0836 mmol), and V-501 (5 mg, 0.0167 mmol) were all combined in a 50 mL round-bottomed flask. The flask was septum-sealed and purged for 40 minutes prior to polymerization in an oil bath at 70 ˚C. The aliquots were taken at pre-determined intervals using degassed syringes and samples were characterized using aqueous SEC and ^{1}H NMR.



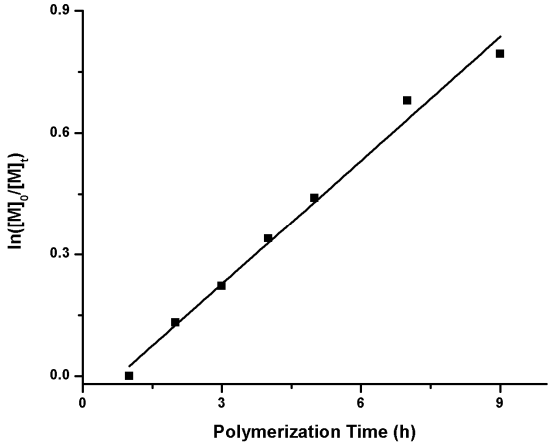
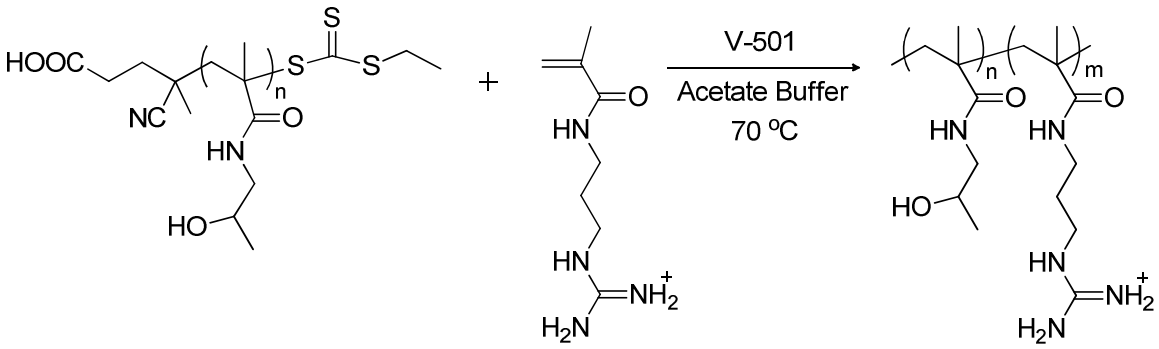


Figure S5. Refractive Index traces (top) and plot of $\ln(M_0/M)$ vs. time of the growing GPMA homopolymer fitted to a linear prediction (bottom).

Synthesis of HPMA MacroCTA:
A 50 mL round-bottomed flask was charged with HPMA (3g, 21.1 mmol) and 20 mL of acetic buffer (pH 5.2, 0.27 M acetic acid and 0.73 M sodium acetate). CEP (15.9 mg, 0.0604 mmol) and V-501 (3.4 mg, 0.0121 mmol) were dissolved in 1 mL of methanol prior to addition to the flask, diluting the mixture to $[M]_0$ = 1.0 M. The flask was then septum-sealed and purged with nitrogen for 40 minutes prior to placing in an oil bath at 70 ˚C for 15 h. A CTA to initiator ratio of $[CTA]_0/[I]_0$ = 5:1 was used with $[M]_0$:[CTA] = 350/1. Samples were characterized using aqueous SEC and ^{1}H NMR.

Synthesis of HPMA-*b*-GPMA Block Copolymer:
A 10 mL round-bottomed flask was charged with HPMA macroCTA (0.313g, 0.00836 mmol), GPMA (43 mg, 0.232 mmol), and 1.2 mL of acetic buffer (pH 5.2, 0.27 M acetic acid and 0.73 M sodium acetate). V-501 (0.661 mg, 0.00236 mmol) was added using a 0.1 wt% stock solution in methanol, diluting the mixture to $[M]_0$ = 1.0 M. A $[CTA]_0/[I]_0$ ratio of 3.5:1 was used with $[M]_0$:[CTA] = 28/1. The solution was purged for 40 minutes with nitrogen prior to overnight polymerization at 70 ˚C. Following polymerization, the sample was dialyzed and lyophilized. Samples were characterized using aqueous SEC and ^{1}H NMR.



Scheme S3. Formation of HPMA-*b*-GPMA copolymers via chain extension of HPMA homopolymer with GPMA.

Synthesis of GPMA-*b*-GPMA Block Copolymer:
To a 10 mL round-bottomed flask was added GPMA (0.312 g, 1.69 mmol), CEP (2.96 mg, 0.0112 mmol), V-501 (0.63 mg, 0.00225 mmol), and 1.7 mL of acetic buffer (pH 5.2, 0.27 M acetic acid and 0.73 M sodium acetate). The CEP and V-501 were both added using 0.1 wt% stock solutions in MeOH, diluting the mixture to $[M]_0$ = 0.85 M. Prior to polymerization at 70 ˚C, the solution was filtered and purged for 45 minutes. A $[CTA]_0/[I]_0$ ratio of 5:1 was used with $[M]_0$:[CTA] = 150/1. The reaction was quenched after 6 h (17 % conversion) by allowing it to cool and exposing it to air. Following this, additional V-501 (0.727 mg, 0.00259 mmol) was added and the reaction was purged for 30 minutes prior to overnight polymerization at 70 ˚C. Samples were characterized using aqueous SEC and ^{1}H NMR.

General Procedure for the Synthesis of FITC-labeled HPMA, GPMA, and HPMA-*b*-GPMA

The free carboxylic acid at the terminus of the RAFT polymers was used to EDC couple an amine containing FITC dye. To a 20-mL scintillation vial was added polymer (30 mg), EDAC (20x excess), and sulfo-NHS (20x excess). The reagents were dissolved in acetate buffer (0.1 M) at pH 6. The vial was set to stir at room temperature for 12 h. FITC (20x excess) was added to the reaction and the vials were wrapped in foil and allowed to stir for 24 h. The samples were then dialyzed against DI water for several days and lyophilized to dryness. Dye conjugation to the terminal carboxyl functionality was quantitative (using an average extinction coefficient for FITC).

Cell Culture

KB cells were incubated and proliferated in RPMI 1640 (Gibco, #27016) cell media containing 10% fetal calf serum (FCS), 100 μg mL^{-1} streptomycin, and 100 unit/mL^{-1} penicillin at 37 ˚C and 4 ˚C in 95% air humidified atmosphere and 5% CO_2.

Cell Treatment with FITC-labeled Polymers for Fluorescence Microscopy

KB cells were seeded on cover glass in a 12-well plate with 1 mL of RPMI 1640 cell media containing 10% FCS for 24 h. Following this, cells were incubated for 2.5 h at both 37 ˚C and 4 ˚C. Polymer concentrations were adjusted in order to normalize fluorescence (HPMA= 10 μL; GPMA= 10 μL; HPMA-*b*-GPMA= 20 μL). After incubation, the cells were washed with 4% paraformaldehyde and washed with phosphate buffer saline (PBS) before imaging. The cover glass was then removed and mounted on 20 μL of 4′,6-diamidino-2-phenylindole (DAPI) mounting gel.

Fluorescence Microscopy

Cell images were taken using a Nikon fluorescence microscope (Eclipse 80i, Plan Fluor 40X/0.75 DIC M/N2 lens) and the images were processed using Image-Pro Plus software.

Cell Treatment with FITC-labeled Polymers for Confocal Fluorescence Microscopy

KB cells and SKOV3 cells were seeded on Lab-Tek™ Chambered Coverglass (Cat. No. 155411) with 200 μL of RPMI 1640 cell media containing 10% FCS for 24 h. Following this, cells were incubated for 1 h at either 37 ˚C or 4 ˚C. Polymer concentrations were adjusted in order to normalize fluorescence (HPMA= 10 μL; GPMA= 10 μL; HPMA-*b*-GPMA= 20 μL). After incubation, the cells were washed with phosphate buffer saline (PBS) three times (each time for 5 minutes at 37 ˚C) before imaging.

Z-stack Confocal Fluorescence Microscopy

Cell images were taken using a Zeiss LSM 510 Meta Confocal Laser Scanning Microscope. Z-stack scanning was performed by adjusting the focal plane from the bottom to the top of the cell in 2 μm increments. The images showing here were chosen from the focal plane around the middle of the cell. From the images, FITC-labeled polymers were clearly internalized into the cell. As expected for a positively charged polymer, both GPMA and HPMA-b-GPMA were seen to bind to the cell membrane as well.

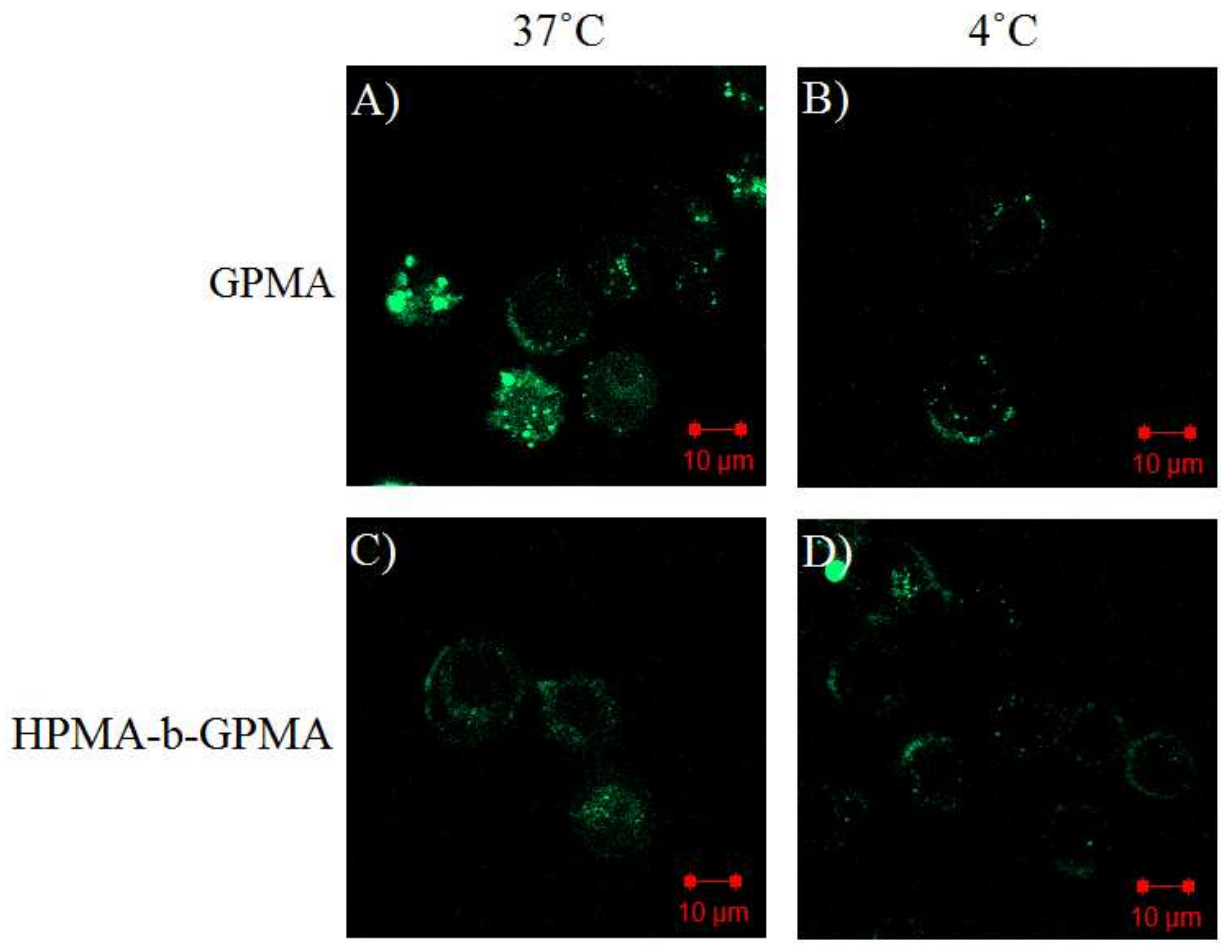


Figure S6. Sectional confocal laser scanning microscopy images from KB cells treated with FITC-labeled polymers at 37 ˚C (A, C) and 4 ˚C (B, D). FITC-labeled GPMA (Mn = 18,100 g/mol) is shown in A and B, and HPMA-b-GPMA (Mn = 39,810 g/mol) is shown in C and D. these images were from 6 µm to 8 µm sections of Z-stack images.

Cell Treatment with FITC-labeled Polymers for Flow Cytometry

KB cells were seeded in a 12-well plate using 1 mL of RPMI 1640 cell media containing 10% FCS for 24 h. Following this, cells were incubated for 2.5 h at both 37 ˚C and 4 ˚C. Polymer concentrations were adjusted in order to normalize fluorescence (HPMA= 10 µL; GPMA= 10 µL; HPMA-*b*-GPMA= 20 µL). Cells were then treated with 200 µL of trypsin for 5 min before centrifuging and washing with PBS. After removing excess PBS, the cells were injected into the flow cytometer (data on the actual flow cytometer). The data was processed using CFlow Plus software.

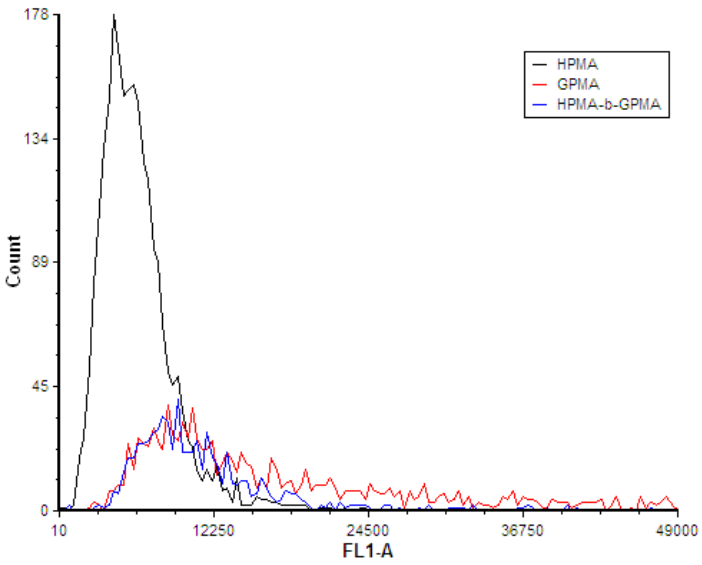


Figure S7A. Flow cytometry of polymers incubated with KB cells for 2.5 h at 4 ˚C showing a shift to higher fluorescence for the GPMA (red) and HPMA-*b*-GPMA (blue) polymers, indicating cell internalization.

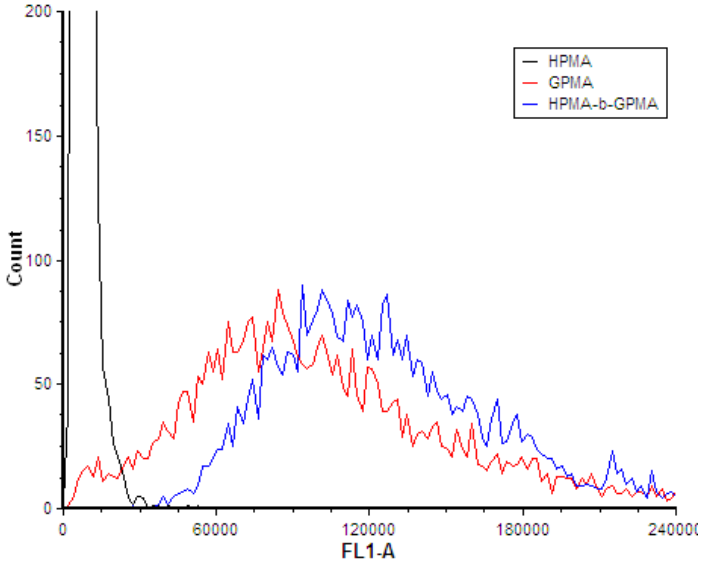


Figure S7B. Flow cytometry of polymers incubated with KB cells for 2.5 h at 37 ˚C revealing a dramatic increase in fluorescence for GPMA homopolymer (red) compared to HPMA homopolymer (black), and an even higher increase in fluorescence for HPMA-*b*-GPMA copolymers, indicating significant cellular uptake.

Table S1. Mean fluorescence and cell counts for the flow cytometry tests conducted at both 37 ˚C and 4 ˚C for 2.5 h.

Polymer	Mean FL1-A 37 ˚C	Cell Count 37 ˚C	Mean FL1-A 4 ˚C	Cell Count 4 ˚C

$PHPMA_{458}$	7500	4700	6000	2400
$PGPMA_{98}$	107000	4000	54800	1400
$P(HPMA_{271}$-*b*-$GPMA_{13})$	131000	4000	12300	700

References

(1) Convertine, A. J.; Benoit, D. S. W.; Duvall, C. L.; Hoffman, A. S.; Stayton, P. S. *J. Controlled Release* **2009,** *133*, 221-229
(2) Spivak, D.; Shea, K. J. *J. Org. Chem.* **1999,** *64*, 4627-4634