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

Nicolas J. Treat,[†] DeeDee Smith,[†] Chengwen Teng,[‡] Joel D. Flores,[†] Brooks A. Abel,[†] Adam W. York,[†] Faqing Huang,[‡] and Charles L. McCormick*[,†,‡]

[†]Department of Polymer Science and [‡]Department of Chemistry and Biochemistry, University of Southern Mississippi, 118 College Drive, Hattiesburg, Mississippi 39406, United States

ⓢ Supporting Information

ABSTRACT: We report the synthesis and controlled radical homopolymerization and block copolymerization of 3-guanidinopropyl methacrylamide (GPMA) utilizing aqueous reversible addition–fragmentation chain transfer (*a*RAFT) polymerization. The resulting homopolymer and block copolymer with *N*-(2-hydroxypropyl) methacrylamide (HPMA) were prepared to mimic the behavior of cell-penetrating peptides (CPPs) and poly(arginine) (>6 units), which have been shown to cross cell membranes. The homopolymerization mediated by 4-cyano-4-(ethylsulfanylthiocarbonylsulfanyl)pentanoic acid (CEP) in aqueous buffer exhibited pseudo-first-order kinetics

Cell penetration

HPMA-b-GPMA Copolymer

and linear growth of molecular weight with conversion. Retention of the "living" thiocarbonylthio ω-end group was demonstrated through successful chain extension of the GPMA macroCTA yielding GPMA$_{37}$-*b*-GPMA$_{61}$ (M_w/M_n = 1.05). Block copolymers of GPMA with the nonimmunogenic, biocompatible HPMA were synthesized yielding HPMA$_{271}$-*b*-GPMA$_{13}$ (M_w/M_n = 1.15). Notably, intracellular uptake was confirmed by fluorescence microscopy, confocal laser scanning microscopy, and flow cytometry experiments after incubation for 2.5 h with KB cells at 4 °C and at 37 °C utilizing FITC-labeled, GPMA-containing copolymers. The observed facility of cellular uptake and the structural control afforded by *a*RAFT polymerization suggest significant potential for these synthetic (co)polymers as drug delivery vehicles in targeted therapies.
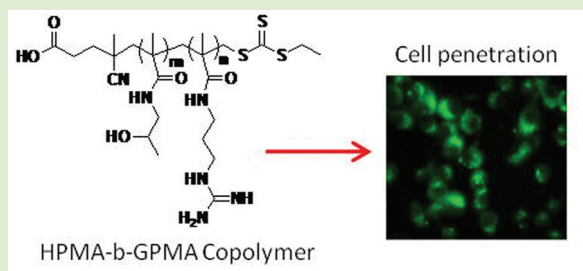
In recent years, there has been extensive research regarding the unique cellular uptake properties of cell-penetrating peptides (CPPs).[1−4] Common CPPs, such as Tat and poly(arginine), are small (<20 nm) and cationic and can cross the plasma membrane of most mammalian cells.[5] Significantly, entry of both Tat and poly(arginine) oligopeptides into cells can occur via an endocytotic-independent pathway, although the precise mechanism is still debated.[5−12] The enhanced cellular uptake of these peptides is reported to depend on the presence of basic amino acid sequences rich in arginine residues and not on peptide secondary structure.[5,12,13] Wender and co-workers have further demonstrated the cell penetrating properties of arginine by synthesizing a D-arginine oligomer (9 units) that exhibited a >100-fold increase in the rate of cellular uptake over Tat$_{49−57}$.[12]

One of the most attractive features of CPPs is their ability to transport macromolecules easily across cellular membranes. Additionally, analysis of in vivo tissue samples reveals uptake into most tissues, including the brain.[2,14] On the basis of observed transmembrane transport alone, modification of synthetic drug delivery vehicles with CPPs appears to hold great promise in targeted therapies. Cellular uptake of synthetic drug delivery vehicles may occur through one of several endocytotic pathways. For efficacious delivery, the internalized vehicle and/or the delivered cargo must escape the endosome prior to lysosomal degradation or exocytosis. Some suggested mechanisms for assisting escape involve membrane disruption

(i.e., proton sponge effect and fusogenic peptides). Another potential strategy for efficacious delivery involves bypassing endocytosis altogether, a process that conceptually would avoid lysosomal degradation and/or exocytosis of the packaged therapeutic. For example, Kopecek and co-workers conjugated a Tat peptide to a biocompatible copolymer that was subsequently internalized into ovarian mammalian cancer cells through both endocytotic and nonendocytotic pathways.[15,16] However, despite the success in cell uptake, the difficulty of the synthesis and the low conjugation efficiency demonstrated the need for a more direct route. Funhoff et al. polymerized a guanidine-containing methacrylate by classical free radical polymerization and condensed plasmid DNA into small polyplexes that successfully transfected COS-7 cells in the absence of serum. In the latter case, however, cellular uptake of the free (uncontrolled) polymer and its complexes was mainly endocytotic in nature rather than via direct cell penetration.[17] Inspired by the work described above and drawing from our previous success at controlled aqueous polymerization, we targeted (co)polymer architecture that might mimic the cell uptake behavior of Tat, poly(arginine), or other guanidine-pendent polyplexes.
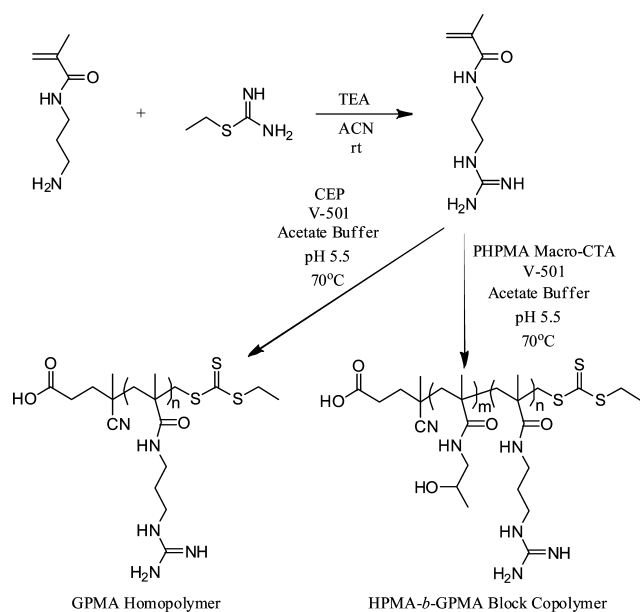
Controlled polymerization techniques now allow tailored block lengths and advanced architectures while maintaining narrow molecular weight distributions. Here we report the aqueous reversible addition− fragmentation chain transfer (aRAFT) polymerization of guanidine-containing monomers directly in water without protecting groups, thus adding an additional synthetic pathway for highly functional systems.[18−22] Previously, RAFT polymerizations have been reported with a variety of functional monomers, including anionic,[23−25] zwitterionic,[26,27] and neutral[28−40] types in both organic and aqueous media.[41−48] For example, our group conducted the initial controlled polymerization of the cationic methacrylamide monomer, N-[3-(dimethylamino)propyl]methacrylamide (DMAPMA), using aqueous media and 4-cyanopentanoic acid dithiobenzoate (CTP) as the chain transfer agent.[18] An acidic environment was necessary to obtain controlled molecular weight (M_n) and low M_w/M_n values.

To provide a controlled synthetic mimic for cell penetration, we first prepared 3-guanidinopropyl methacrylamide (GPMA) and subsequently conducted its polymerization via aRAFT in an acetate buffer solution mediated by 4-cyano-4-(ethylsulfanylthiocarbonylsulfanyl)pentanoic acid (CEP) as the chain transfer agent. The polymerization showed linear molecular weight dependence with conversion, yielding control over both M_n and M_w/M_n. Chain extension of the polyGPMA macroCTA was successfully accomplished by adding GPMA, as was block copolymerization by adding GPMA to the polyHPMA macroCTA. HPMA was chosen as a comonomer because polyHPMA has the reported attributes of being bio-compatible, nonimmunogenic, and sufficiently hydrophilic to promote the enhanced permeability and retention (EPR) effect that allows accumulation within tumoral tissue.[49,50]

In our work, we adapted a one-step approach to the synthesis of GPMA first reported by Shea et al.[51] We utilized aminopropyl methacrylamide (APMA) and 2-ethyl-2-thiopseudourea hydro-bromide (Scheme 1) to prepare the methacrylamide monomer in 72% yield (full experimental description and characterization, Schemes S1−S3, and Figures S1−S4 can be found in the Supporting Information). The guanidinium group, while not a strong nucleophile, is very basic with the guanidinium cation having a pK_a of ~13. The polymerization kinetics, shown in Figure 1, were determined using CEP as the chain transfer agent
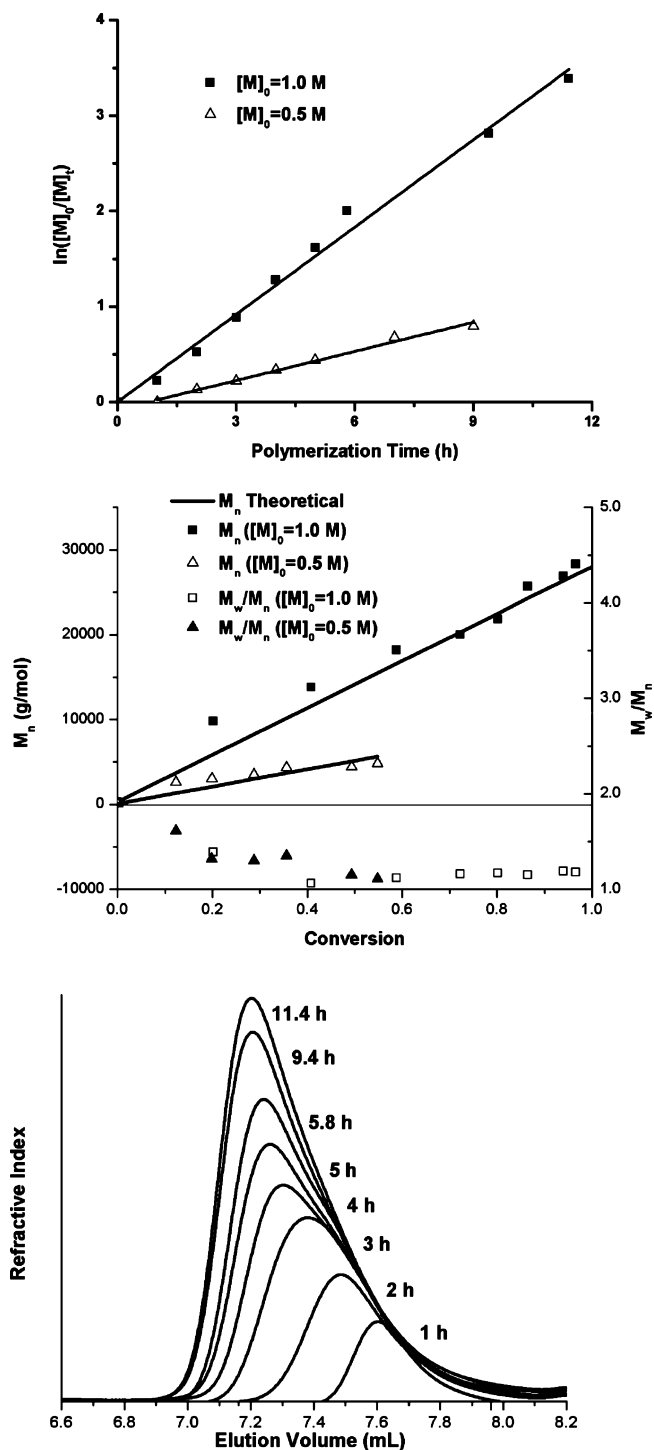
Scheme 1. Synthesis of 3-Guanidinopropyl Methacrylamide (GPMA) and Subsequent aRAFT Polymerization of the Monomer To Form a GPMA Homopolymer and HPMA Block Copolymer

Figure 1. Plot of $\ln([M]_0/[M]_t)$ vs conversion of the growing GPMA homopolymer fitted to a linear prediction (top). Plot of M_n vs conversion of the growing GPMA homopolymer with M_n(theory) and M_w/M_n (middle) and refractive index traces (bottom).

and V-501 as the initiator at two initial monomer concentrations (0.5 and 1.0 M). The linearity of the kinetic plots (Figure 1, top)

up to 97% conversion demonstrates the pseudo-first-order behavior of the polymerization. The refractive index traces were symmetrical and shifted to lower elution volumes as the reaction proceeded (Figure 1, bottom). As often observed for CEP-mediated RAFT polymerization of acrylamido monomers, after an early initialization period, experimental molecular weights (close to those theoretically predicted) and narrow M_w/M_n values were observed with conversion (Figure 1, middle, and Table 1).

Table 1. GPMA Homopolymers Synthesized at Initial Monomer Concentrations ($[M]_0$) of 0.5 and 1.0 M in Acetate Buffer at 70 °C with V-501 as a Free Radical Initiator and CEP as a Chain Transfer Agent

time (h)	$[M]_0$ (M)	conversion (%)	M_n(expt)	M_n(theory)	M_w/M_n
1	0.5	0	300	300	–
2	0.5	12	2800	1500	1.61
3	0.5	20	3200	2300	1.32
4	0.5	29	3700	3200	1.30
5	0.5	36	4500	3900	1.35
7	0.5	49	4600	5200	1.15
9	0.5	55	5000	5800	1.11
1	1.0	20	9800	5900	1.39
2	1.0	41	13800	11600	1.06
3	1.0	59	18200	16600	1.12
4	1.0	72	20000	20300	1.16
5	1.0	80	21800	22500	1.17
5.8	1.0	87	25700	24300	1.15
9.4	1.0	94	26900	26400	1.19
11.4	1.0	97	28300	27100	1.18

To further verify the livingness of the aRAFT polymerization of the GPMA monomer, we conducted blocking experiments with a PGPMA macroCTA (Figure 2, top) and a PHPMA macroCTA (Figure 2, bottom). PGPMA macroCTA was synthesized in acetate buffer at pH 5.2 with CEP as the chain transfer agent and V-501 as the initiator. The polymerization was quenched after 6 h (17% conversion), and an aliquot was taken for GPC analysis (Figure 2, top). Additional V-501 was added, and the reaction was allowed to proceed overnight (98% conversion). The shift in elution volume (Figure 2, top) of the GPMA$_{37}$-b-GPMA$_{61}$ homopolymer shows that the chain ends remained active with no detectable hydrolysis or aminolysis.

An HPMA-b-GPMA copolymer was also synthesized to prepare copolymers that structurally mimic CPPs or poly-(arginine). The HPMA macroCTA was synthesized as previously reported.[21] Following purification via dialysis and lyophilization, the macroCTA was chain extended with GPMA using V-501 as the initiator. Because poly(arginine) exhibits optimal cellular uptake at lower (>6 repeat units)[8,9] segmental lengths, block copolymers consisting of a long HPMA block ($X_n = 271$) and a short GPMA block length ($X_n = 13$) were targeted. Chain extension was successful as demonstrated by the shift in elution volume (Figure 2, bottom) of the block copolymer.

The ability of GPMA$_{98}$ and HPMA$_{271}$-b-GPMA$_{13}$ to enter cells via both endocytotic and nonendocytotic pathways was probed by incubating the polymers with KB cells at 37 and 4 °C. At 37 °C, all energy-dependent endocytotic pathways (clathrin-mediated endocytosis, caveolae, macropinocytosis, and phagocytosis) are operational.[52] However, at 4 °C, ATP production is slowed considerably, and these pathways are thus inhibited.[53] Therefore, uptake of any
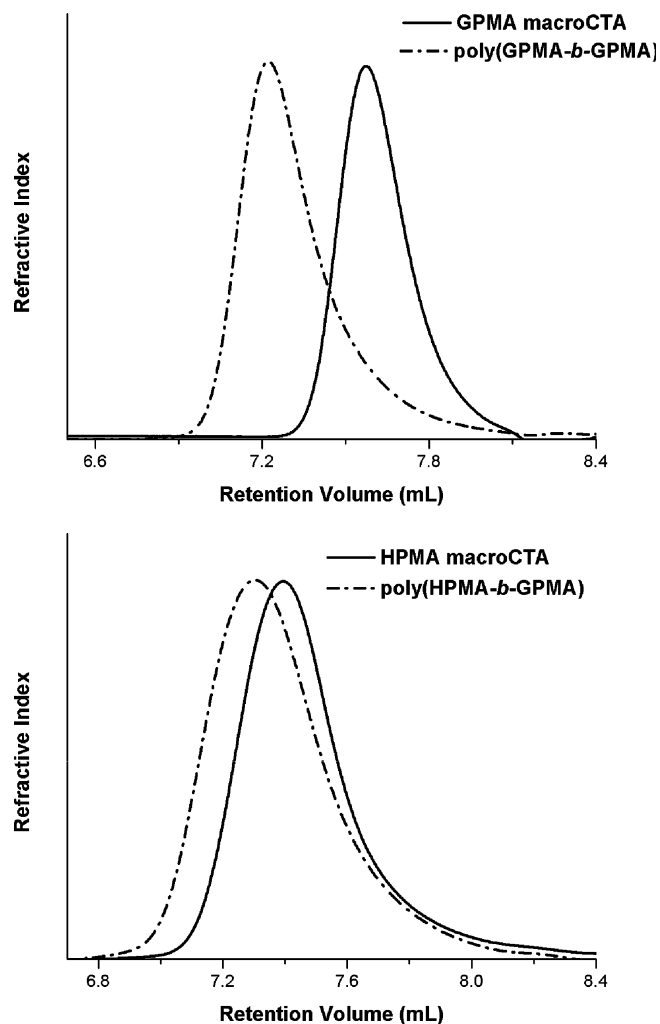

Figure 2. Aqueous size exclusion chromatography trace of GPMA macroCTA chain extension with GPMA to form GPMA-b-GPMA copolymers (top). Aqueous size exclusion chromatography trace of HPMA macroCTA chain extension with GPMA to form HPMA-b-GPMA copolymer (bottom).

macromolecular structure should occur via an alternative uptake mechanism. Polymers were labeled with an amine-containing FITC dye via the carboxylic acid end group of the polymer using EDC coupling with sulfo-NHS in acetate buffer at pH 6. The polymer samples (HPMA$_{458}$, GPMA$_{98}$, and HPMA$_{271}$-b-GPMA$_{13}$) were incubated with KB cells for 2.5 h at 37 and 4 °C. Each polymer sample was examined using fluorescence microscopy (Figure 3), confocal scanning laser microscopy (Figure S6 of the Supporting Information), and flow cytometry (Figure S7 of the Supporting Information). As expected, the HPMA homopolymer did not enter cells at 37 or 4 °C for the short incubation time period (panels A and B of Figure 3) because there is no moiety for direct uptake.[15,16] Both the GPMA homopolymer and the cell-penetrating peptide mimic (HPMA$_{271}$-b-GPMA$_{13}$) showed significant uptake in KB cells after incubation for 2.5 h at 37 and 4 °C. In addition, flow cytometry results indicated that the HPMA$_{271}$-b-GPMA$_{13}$ copolymer had an increased level of uptake compared to the GPMA homopolymer at 37 °C (Figure S7B of the Supporting Information). Fluorescence microscopy and flow cytometry of KB cells incubated with the polymer at 4 °C showed both GPMA$_{98}$ and HPMA$_{271}$-b-GPMA$_{13}$ entering cells (panels D and F of Figure 3 and Figure S7B of the Supporting Information). The cell
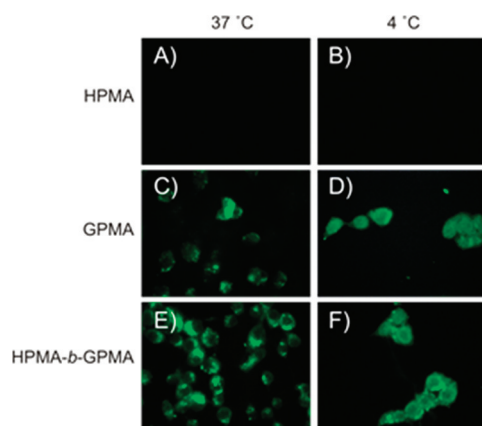
Figure 3. Fluorescent microscopy images of FITC-labeled polymers at 37 °C (A, C, and E) and 4 °C (B, D, and F) in KB cells. FITC-labeled HPMA (M_n = 60000 g/mol) is shown in panels A and B with no fluorescence. GPMA (M_n = 18100 g/mol) is shown in panels C and D, with transfection of cells at 37 and 4 °C. HPMA-b-GPMA (M_n = 39810 g/mol) is shown in panels E and F, with transfection of cells at 37 and 4 °C.

count and mean fluorescence for these cells were significantly lower than those observed for tests conducted at 37 °C (Table S1 of the Supporting Information). To confirm the entry of the polymer into the cells, cellular cross sections from top to bottom were examined by Z-stack confocal laser scanning microscopy (Figure S6 of the Supporting Information). Together, these results suggest that GPMA$_{98}$ and HPMA$_{271}$-b-GPMA$_{13}$ may enter the cell through both endocytotic and energy-independent pathways. Although not the primary focus of this work, further studies will be necessary to fully understand the cellular uptake behavior and capabilities of these synthetic copolymers.

In conclusion, we successfully synthesized homopolymers and block copolymers of a guanidinium-containing methacrylamide monomer using aRAFT polymerization. The block copolymer HPMA$_{271}$-b-GPMA$_{13}$ and homopolymer GPMA$_{98}$ were incubated with KB cells at 37 and 4 °C to investigate the mechanism of uptake. Fluorescence microscopy and flow cytometry results indicate intracellular uptake via both endocytotic and energy-independent pathways. The ability to tailor precise architectures, the molecular weight, and molecular weight distribution directly in water opens the door for guanidinium-functional polymers to be used as prodrugs, in gene delivery, or for other applications as advanced biomaterials.[22]

ASSOCIATED CONTENT

⑤ Supporting Information

Experimental details and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author
*E-mail: Charles.McCormick@usm.edu.

Notes
Paper 153 in a series entitled "Water-Soluble Polymers".

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

REFERENCES

(1) Derossi, D.; Joliot, A. H.; Chassaing, G.; Prochiantz, A. *J. Biol. Chem.* **1994**, *269*, 10444−10450.
(2) Snyder, E. L.; Dowdy, S. F. *Pharm. Res.* **2004**, *21*, 389−393.
(3) Vives, E. *J. Controlled Release* **2005**, *109*, 77−85.
(4) Vives, E.; Brodin, P.; Lebleu, B. *J. Biol. Chem.* **1997**, *272*, 16010−16017.
(5) Silhol, M.; Tyagi, M.; Giacca, M.; Lebleu, B.; Vives, E. *Eur. J. Biochem.* **2002**, *269*, 494−501.
(6) Fawell, S.; Seery, J.; Daikh, Y.; Moore, C.; Chen, L. L.; Pepinsky, B.; Barsoum, J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 664−668.
(7) Futaki, S. *Int. J. Pharm.* **2002**, *245*, 1−7.
(8) Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2001**, *276*, 5836−5840.
(9) Mitchell, D. J.; Kim, D. T.; Steinman, L.; Fathman, C. G.; Rothbard, J. B. *J. Pept. Res.* **2000**, *56*, 318−325.
(10) Suzuki, T.; Futaki, S.; Niwa, M.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2002**, *277*, 2437−2443.
(11) Vives, E.; Granier, C.; Prevot, P.; Lebleu, B. *Lett. Pept. Sci.* **1997**, *4*, 429−436.
(12) Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13003−13008.
(13) Pouton, C. W.; Lucas, P.; Thomas, B. J.; Uduehi, A. N.; Milroy, D. A.; Moss, S. H. *J. Controlled Release* **1998**, *53*, 289−299.
(14) Rousselle, C.; Clair, P.; Lefauconnier, J. M.; Kaczorek, M.; Scherrmann, J. M.; Temsamani, J. *Mol. Pharmacol.* **2000**, *57*, 679−686.
(15) Nori, A.; Jensen, K. D.; Tijerina, M.; Kopeckova, P.; Kopecek, J. *J. Controlled Release* **2003**, *91*, 53−59.
(16) Nori, A.; Jensen, K. D.; Tijerina, M.; Kopeckova, P.; Kopecek, J. *Bioconjugate Chem.* **2003**, *14*, 44−50.
(17) Funhoff, A. M.; Van Nostrum, C. F.; Lok, M. C.; Fretz, M. M.; Crommelin, D. J. A.; Hennink, W. E. *Bioconjugate Chem.* **2004**, *15*, 1212−1220.
(18) Scales, C. W.; Huang, F.; Li, N.; Vasilieva, Y. A.; Ray, J.; Convertine, A. J.; McCormick, C. L. *Macromolecules* **2006**, *39*, 6871−6881.
(19) York, A. W.; Huang, F.; McCormick, C. L. *Biomacromolecules* **2010**, *11*, 505−514.
(20) York, A. W.; Kirkland, S. E.; McCormick, C. L. *Adv. Drug Delivery Rev.* **2008**, *60*, 1018−1036.
(21) York, A. W.; Zhang, Y.; Holley, A. C.; Guo, Y.; Huang, F.; McCormick, C. L. *Biomacromolecules* **2009**, *10*, 936−943.
(22) Hunt, J. N.; Feldman, K. E.; Lynd, N. A.; Deek, J.; Campos, L. M.; Spruell, J. M.; Hernandez, B. M.; Kramer, E. J.; Hawker, C. J. *Adv. Mater.* **2011**, *23*, 2327−2331.
(23) Convertine, A. J.; Benoit, D. S. W.; Duvall, C. L.; Hoffman, A. S.; Stayton, P. S. *J. Controlled Release* **2009**, *133*, 221−229.
(24) Sumerlin, B. S.; Donovan, M. S.; Mitsukami, Y.; Lowe, A. B.; McCormick, C. L. *Macromolecules* **2001**, *34*, 6561−6564.
(25) Sumerlin, B. S.; Lowe, A. B.; Thomas, D. B.; McCormick, C. L. *Macromolecules* **2003**, *36*, 5982−5987.
(26) Donovan, M. S.; Lowe, A. B.; Sanford, T. A.; McCormick, C. L. *J. Polym. Sci., Part A: Polym. Chem.* **2003**, *41*, 1262−1281.
(27) Yusa, S.; Shimada, Y.; Mitsukami, Y.; Yamamoto, T.; Morishima, Y. *Macromolecules* **2003**, *36*, 4208−4215.
(28) An, Z.; Shi, Q.; Tang, W.; Tsung, C.-K.; Hawker, C. J.; Stucky, G. D. *J. Am. Chem. Soc.* **2007**, *129*, 14493−14499.
(29) Bernard, J.; Hao, X.; Davis, T. P.; Barner-Kowollik, C.; Stenzel, M. H. *Biomacromolecules* **2006**, *7*, 232−238.
(30) Boyer, C.; Liu, J.; Wong, L.; Tippett, M.; Bulmus, V.; Davis, T. P. *J. Polym. Sci., Part A: Polym. Chem.* **2008**, *46*, 7207−7224.

(31) Convertine, A. J.; Lokitz, B. S.; Lowe, A. B.; Scales, C. W.; Myrick, L. J.; McCormick, C. L. *Macromol. Rapid Commun.* **2005**, *26*, 791−795.

(32) Donovan, M. S.; Sanford, T. A.; Lowe, A. B.; Sumerlin, B. S.; Mitsukami, Y.; McCormick, C. L. *Macromolecules* **2002**, *35*, 4570−4572.

(33) Favier, A.; Charreyre, M.-T.; Pichot, C. *Polymer* **2004**, *45*, 8661−8674.

(34) Joso, R.; Stenzel, M. H.; Davis, T. P.; Barner-Kowollik, C.; Barner, L. *Aust. J. Chem.* **2005**, *58*, 468−471.

(35) Kakwere, H.; Chun, C. K. Y.; Jolliffe, K. A.; Payne, R. J.; Perrier, S. *Chem. Commun.* **2010**, *46*, 2188−2190.

(36) Millard, P.-E.; Barner, L.; Reinhardt, J.; Buchmeiser, M. R.; Barner-Kowollik, C.; Mueller, A. H. E. *Polymer* **2010**, *51*, 4319−4328.

(37) Mori, H.; Kato, I.; Matsuyama, M.; Endo, T. *Macromolecules* **2008**, *41*, 5604−5615.

(38) Ouyang, L.; Wang, L.; Schork, F. J. *Polymer* **2011**, *52*, 63−67.

(39) Ouyang, L.; Wang, L.; Schork, F. J. *Macromol. Chem. Phys.* **2010**, *211*, 1977−1983.

(40) Thomas, D. B.; Convertine, A. J.; Myrick, L. J.; Scales, C. W.; Smith, A. E.; Lowe, A. B.; Vasilieva, Y. A.; Ayres, N.; McCormick, C. L. *Macromolecules* **2004**, *37*, 8941−8950.

(41) Favier, A.; Charreyere, M.-T.; Chaumont, P.; Pichot, C. *Macromolecules* **2002**, *35*, 8271−8280.

(42) Lowe, A. B.; McCormick, C. L. *Prog. Polym. Sci.* **2007**, *32*, 283−351.

(43) McCormick, C. L.; Lowe, A. B. *Acc. Chem. Res.* **2004**, *37*, 312−325.

(44) Moad, G.; Rizzardo, E.; Thang, S. H. *Aust. J. Chem.* **2006**, *59*, 669−692.

(45) Moad, G.; Rizzardo, E.; Thang, S. H. *Polymer* **2008**, *49*, 1079−1131.

(46) Boyer, C.; Granville, A.; Davis, T. P.; Bulmus, V. *J. Polym. Sci., Part A: Polym. Chem.* **2009**, *47*, 3773−3794.

(47) Min, E. H.; Ting, S. R. S.; Billon, L.; Stenzel, M. H. *J. Polym. Sci., Part A: Polym. Chem.* **2010**, *48*, 3440−3455.

(48) Liu, Z.; Hu, J.; Sun, J.; He, G.; Li, Y.; Zhang, G. *J. Polym. Sci., Part A: Polym. Chem.* **2010**, *48*, 3573−3586.

(49) Kopecek, J.; Kopeckova, P. *Adv. Drug Delivery Rev.* **2010**, *62*, 122−149.

(50) Matsumura, Y.; Maeda, H. *Cancer Res.* **1986**, *46*, 6387−6392.

(51) Spivak, D.; Shea, K. J. *J. Org. Chem.* **1999**, *64*, 4627−4634.

(52) Jones, A. T. *J. Cell. Mol. Med.* **2007**, *11*, 670−684.

(53) Duncan, R.; Lloyd, J. B. *Biochim. Biophys. Acta* **1978**, *544*, 647−655.