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Thermoresponsive Micelles from Double LCST-Poly(3-methyl-*N*-vinylcaprolactam) Block Copolymers for Cancer Therapy

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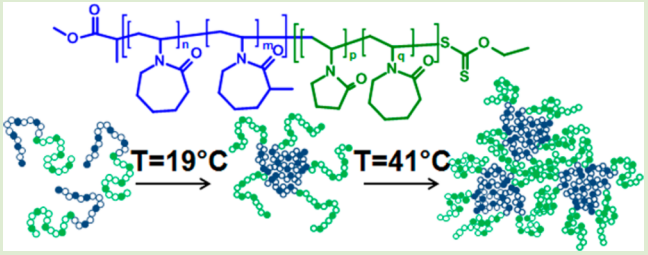
Supporting Information

ABSTRACT: We present synthesis and assembly of novel thermoresponsive block copolymers with double LCST precisely controlled within the physiological temperature range. Two separate phase transition temperatures were achieved by RAFT polymerization of structurally similar monomers with varied hydrophobicity. The LCST1 was varied from 19 to 27 °C by copolymerization of *N*-vinylcaprolactam with a novel hydrophobic monomer, 3-methyl-*N*-vinylcaprolactam, while the LCST2 at 41−42 °C was attained by copolymerization of *N*-vinylcaprolactam with hydrophilic *N*-vinylpyrrolidone. The LCST1 facilitates micelle formation and entrapment of anticancer drug doxorubicin or hydrophobic dye Nile Red into the micelle core surrounded with hydrophilic yet temperature-sensitive corona. The LCST2 induces collapse of the micelle corona and the consequent drug release. The second elevated temperature is typical for tumors and can trigger the drug-loaded micelle aggregation/accumulation within the tumor resulting in the enhanced passive targeting.



Various strategies are used to improve tumor targeting, including low pH and leaky cancerous vasculature.[1−5] The pathological tissues often have a local temperature elevated up to 40−42 °C compared to unaffected organs due to high glycolysis rate and fast proliferation.[6]

Polymer micelles, assembled in aqueous media from amphiphilic block copolymers, have been developed for anticancer drug delivery as their size (40−200 nm) allow their preferential accumulation at tumor tissues via the enhanced permeability and retention used for passive targeting.[7,8] Stimuli-responsive micelles are able to change their morphologies upon external stimuli and release cargo in a controlled way.[9−15] For thermosensitive micelles, enhanced therapeutic effect can be achieved when the micelle size is increased >200 nm due to micelle aggregation within the tumor vascularity. The temperature-triggered micelle aggregation can be attained in tumors because of elevated internal temperature and by heat externally guided on compromised tissues with focused ultrasound and hyperthermia.[7]

Temperature-responsive (lower critical solution temperature) LCST polymers can be used to design either the micelle core which forms at T > LCST or the micelle hydrophilic corona.[16,17] The former has a limited use with thermal therapies as it requires temperature decrease to destabilize the core and release the drug, while in the latter, drug release is achieved from the hydrophobic core upon the collapse of the hydrated corona segments at T > LCST.[18]

Poly(*N*-vinylcaprolactam) (PVC) is a thermoresponsive polymer with a coil-to-globule transition at 36−50 °C, which is tunable, depending on molar mass and concentration.[19] PVC is known for its excellent biocompatibility, stability against hydrolysis, and complexation ability.[20] Lately, the development of controlled polymerization techniques has enabled synthesis of a variety of well-defined PVC-based amphiphilic and double-hydrophilic block copolymers.[21−25] Despite considerable interest in amphiphilic temperature-responsive block copolymers, copolymers with double LCST have been rarely explored and mostly limited to the systems outside the physiological temperature range.[22−27] One of the reasons is large compositional heterogeneity of thermoresponsive blocks typically used for micelle assembly.

Herein, we demonstrate the first example of thermoresponsive poly(3-methyl-*N*-vinylcaprolactam)-based diblock copolymers with two precisely controlled LCSTs within the physiological temperature range (Figure 1). These separate phase transition temperatures are attained by RAFT polymerization of structurally similar monomers with varied hydrophobicity. The first LCST1 ranging from 19 to 27 °C facilitates micelle formation and drug entrapment in the hydrophobic micelle core surrounded with hydrophilic yet temperature-sensitive corona (Figure 2a). The second phase transition temperature is because of the micelle corona segments and is designed to be at 41−42 °C. The temperature-induced collapse of polymer blocks in the micelle corona at the LCST2 leads to an aggregation of the drug-loaded micelles into larger structures followed by drug release (Figure 2a). In addition, the second elevated temperature can trigger the loaded micelle aggrega-

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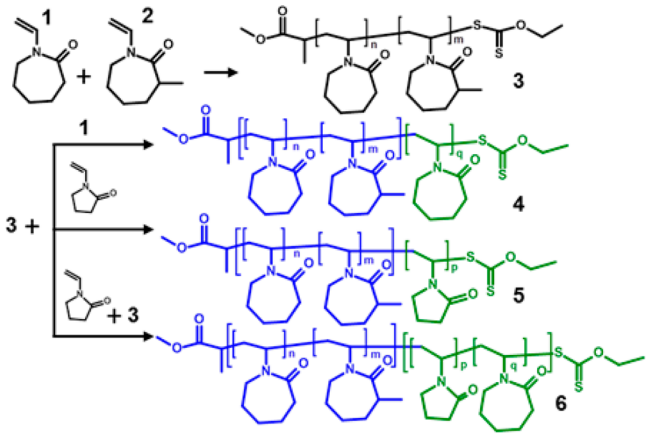

Figure 1. RAFT copolymerization of **1** and **2** to produce temperature-responsive RAFT macroinitiator **3** for thermosensitive **4** and **5** and novel **6** diblock copolymers.

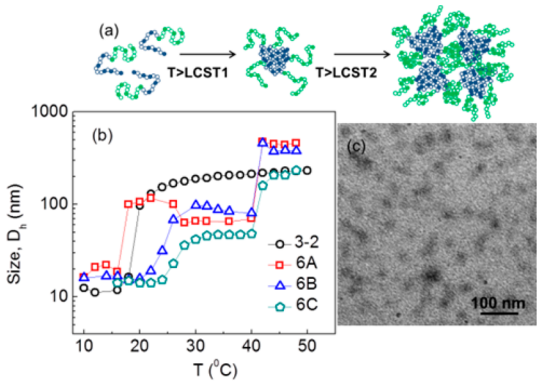

Figure 2. (a) Temperature-triggered self-assembly of diblock copolymers **6** into the micelles at LCST1 followed by formation of micelle aggregates at the second block LCST2. (b) Temperature-dependent hydrodynamic size changes of **3**-**2** and its diblock copolymers **6**. (c) TEM image of micelles prepared from **6C** at 30 °C.

tion/accumulation within the tumor resulting in the enhanced passive targeting.

To obtain the block copolymers with two LCSTs in the physiological temperature range, poly(*N*-vinylcaprolactam) (PVC)-based diblock copolymers were designed and synthesized (Figure 1). Since the LCST of PVC is above room temperature, no stable PVC micelles can be produced under these conditions. To increase the molecular hydrophobicity of the PVC chain and obtain an LCST1 < 30 °C, the first responsive block was synthesized by copolymerizing *N*-vinylcaprolactam (VC; Figure 1; **1**) with a new monomer, 3-methyl-*N*-vinylcaprolactam (MVC; Figure 1; **2**) using statistical RAFT polymerization which resulted in poly(3-methyl-*N*-vinylcaprolactam)-*co*-(*N*-vinylcaprolactam) copolymer, P(MVC-*co*-VC) (Figure 1; **3**). The hydrophilic yet responsive second block of PVC was further synthesized using P(MVC-*co*-VC) as the RAFT macroinitiator (Figure 1; **4**). To increase the hydrophilicity of the PVC-based micelle corona and achieve the second LCST2 at a slightly elevated physiological temperature of ∼40 °C, hydrophilic monomer *N*-vinylpyrrolidone (VP) was used (Figure 1; **5**, **6**).

When PVC is connected to another block, the LCST of the resultant block copolymer is highly dependent on the hydrophilicity of the following block, which enables tuning the copolymer LCST to that useful in biomedical area.[22,23,25,26] We have earlier demonstrated that the LCST of PVC-*b*-PVP block copolymers can be varied from 46 to 38 °C by changing the length of PVC or of the hydrophilic PVP.[20b] Kermagoret et al., reported lately on PVC-based copolymers with PVC as the first responsive block followed by copolymerization of *N*-vinylamide or vinylesters with the rest of VC as the second block.[22,27] Those PVC copolymers had the LCST1 in the range from 35 to 52 °C, while the LCST2 was greater than 70 °C. The high hydrophilicity and a large compositional heterogeneity of the second block led to the high values of LCST1 (>50 °C) and LCST2 (>70 °C),[22] which may hinder the use of those polymers in biomedical field.

In this work, we maintained the minimal compositional difference between the VC blocks synthesizing MVC monomer which is structurally similar to VC. To obtain MVC, the α-position of VC carbonyl group was methylated with methyl iodide. The ^{1}H NMR analysis of MVC confirmed the presence of the characteristic peak of the proton at 1.16 ppm (Figure S1). In contrast to recently reported *N*-vinyl lactam monomers modified with *tert*-butoxycarbonyl groups,[28] we found that methyl group significantly increased the hydrophobicity of the corresponding PMVC homopolymer (M_w = 10000 g/mol, $Đ$ = 1.23, Figure S1), which was not soluble in water even at T = 0 °C. Using statistical RAFT polymerization of MVC and VC with the MVC/VC feeding ratio of 3:1, the novel P(MVC-*co*-VC) macroinitiator with M_n = 10400 g/mol and $Đ$ = 1.18 was synthesized (Figure 1, **3**; Table 1, **3**-**1**). We found similar reactivity ratio of MVC and VC in the copolymerization using ^{1}H NMR analysis of the remaining monomers and by calculating the yield of the reaction. The copolymer had the coil-to-globule transition at 20 °C when large 374 ± 34 nm aggregates were formed in the temperature-dependent size measurements using DLS (Figure 2b; Table 1). The P(MVC-

Table 1. Phase Transition Temperatures, Hydrodynamic Sizes, and CMC of the Copolymers

#	copolymer	T_{c1},[a] °C	D_h1,[b] nm	T_{c2},[a] °C	D_h2,[b] nm	M_n,[c] g mol^{-1}	$Đ$	CMC, mg mL^{-1}
3-1	P(MVC-*co*-VC)	20	374 ± 34			10400	1.18	
4	P(MVC-*co*-VC)-*b*-PVC	24	34 ± 3	30	323 ± 48	61500	1.32	0.01
5	P(MVC-*co*-VC)-*b*-PVP	28	34 ± 4			32400	1.40	0.007
3-2	P(MVC-*co*-VC)	19	210 ± 17			12500	1.15	
6	P(MVC-*co*-VC)-*b*-P(VC-*co*-VP)-A	19	70 ± 4	41	455 ± 14	17000	1.44	0.002
6	P(MVC-*co*-VC)-*b*-P(VC-*co*-VP)-B	24	80 ± 7	41	393 ± 37	28000	1.46	0.003
6	P(MVC-*co*-VC)-*b*-P(VC-*co*-VP)-C	27	48 ± 2	42	226 ± 23	31200	1.41	0.006

[a]Critical temperatures, T_c, were calculated as the inflection point of the temperature-dependent size curves obtained by DLS in which size is a function of temperature. [b]The micelle size is an average hydrodynamic diameter measured at the corresponding inflection point using DLS. [c]The number-average molecular weights were obtained with GPC using polystyrene standards.

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co-VC) was further polymerized with VC or VP to prepare P(MVC-*co*-VC)-*b*-PVC and P(MVC-*co*-VC)-*b*-PVP by the sequential RAFT polymerization of **3**-**1** (Table 1).

The hydrophilicity of the second block can significantly increase the LCST1 of the "micelle core" block as observed earlier for PVC-*b*-PVP and for PVC-*b*-P(methylvinylacetamide-*co*-VC) block copolymers.[20b,22] Indeed, we found the second highly hydrophilic PVP block resulted in an increased phase transition temperature of the copolymer from 20 to 28 °C, while less hydrophilic PVC block led to a smaller increase in LCST1 from 20 to 24 °C and the appearance of the LCST2 = 30 °C (Table 1; Figures S2 and S3). In both cases, the micelles of ∼34 nm were formed at T > LCST1.

To decrease the LCST to 19 °C, a larger molecular weight P(MVC-*co*-VC) copolymer (M_n = 12500 g/mol, $Đ$ = 1.15) was synthesized (Table 1, Figures 2b and S4) and copolymerized further using the VP/VC feeding ratio of 3:1, to result in P(MVC-*co*-VC)-*b*-P(VP-*co*-VC) block copolymers (Figure 1, **6**). After the growth of the second responsive block P(VC-*co*-VP) for 4 h, the M_n of the copolymer increased from 12500 to 17000 g/mol (Table 1, **6A**). Figure 2b shows that all three solutions of block copolymer **6** in DI water demonstrate three T-dependent size regions with two size transitions in the range of 19−41 °C upon heating from 10 to 50 °C as analyzed by DLS.

In the first region, the hydrodynamic size was ∼10 nm, indicating that both copolymer blocks were molecularly dissolved. The increased hydrodynamic diameter to 70 ± 4 nm at T > 18 °C for P(MVC-*co*-VC)-*b*-P(VC-*co*-VP)-A indicated the micelle assembly because of dehydration of the P(MVC-*co*-VC) segments. The micelle size persisted until T > 40 °C when larger ∼400 nm aggregates formed because of the P(VC-*co*-VP) micelle corona dehydration leading to the micelle aggregation (Table 1). Compared to P(MVC-*co*-VC)-*b*-PVC, the difference between the two critical temperatures is extended ($T_{c2} - T_{c1}$ = 6 °C for **4** vs $T_{c2} - T_{c1}$ = 22 °C for **6A**) ensuring clear separation of the micelle and micelle aggregates phases (Table 1). The first T_{c1} was tuned from 19 to 24 and to 27 °C by increasing the molecular weight of the second P(VC-*co*-VP) block via the increased polymerization time from 4 (**6A**) to 8 (**6B**) and 12 (**6C**) hours, respectively, resulting in M_n of 28000 (**6B**) and 31200 g/mol (**6C**), respectively (Table 1; Figure S5). The overall increase in second block hydrophilicity yielded the LCST1 at higher temperatures with no significant change in the LCST2 (Table 1). The micelle formation was confirmed by transmission electron microscopy (TEM) analysis (Figure 2c). The dried micelles assembled from **6C** at 30 °C and observed by TEM were spherical with the smaller average size of 27 ± 3 nm, which is due to their collapse caused by the electron beam.

AFM analysis of **6A** and **6C** solutions exposed to 25 and 35 °C confirmed the presence of micellar structures at both temperatures for **6A** (Figure 3a,b) and only at 35 °C for **6C** (Figure 3c,d). These results are in excellent agreement with the corresponding LCST1 values for the block copolymers (Figure 2b, Table 1).

The critical micelle concentration (CMC) values for **6A**, **6B**, and **6C** copolymers increased with increasing the hydrophilic block length and were 0.002, 0.003, and 0.006 mg/mL, respectively, as measured with fluorescence spectroscopy (Figure S6). These CMCs are much lower than those found for other PVC-based micelles falling in the range from 0.01 to 0.07 mg/mL,[29,30] which demonstrates the superior stability of the micelles reported here.

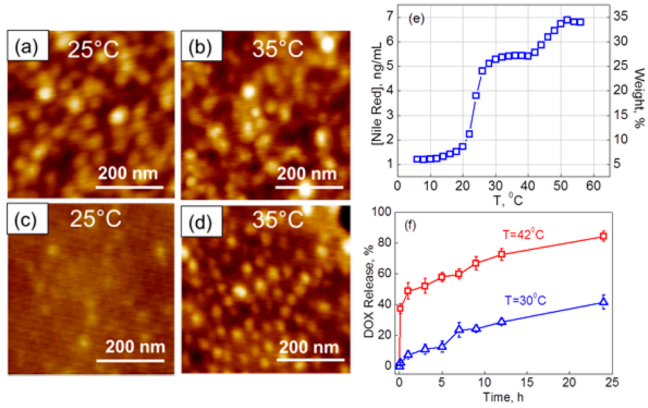

Figure 3. AFM topography images of (a, b) **6A** and (c, d) **6C** aqueous solutions exposed to 25 and 35 °C; z-scale is 18 nm. (e) The NR amount loaded into **6C** micelles at various temperatures, and (f) the DOX release from **6C** micelles at 30 and 42 °C.

The P(MVC-*co*-VC)-*b*-P(VP-*co*-VC) micelles were then loaded with Nile Red (NR) as a model hydrophobic cargo. Temperature-dependent increase in NR fluorescence measured with fluorometry was observed for **6C** at T = 26 °C (Figure S7) at 610 nm due to entrapment of the dye in the micelle hydrophobic P(MVC-*co*-VC) core. The intensity was stable for at least 24 h at this temperature and additionally increased at T > 40 °C due to micelle aggregation (Figure S7c). Compared to 8 wt % loading capacity for PVC-*b*-PEG-FA micelles reported earlier,[29] the P(MVC-*co*-VC)-*b*-P(VC-*co*-VP)-C self-assemblies demonstrated NR loading of 27% (w/w) at T = 26 °C and up to 35% at T = 50 °C. The second phase transition temperature for the NR-loaded micelles appeared at a slightly higher temperature (46 °C) than that for the empty micelles (42 °C) which is because of the hydrophobic interactions between the dye and copolymer chains that weaken the chain−chain interactions and shift the LCST of the corona segments toward a higher temperature (Figure 3e). Repeated heating−cooling cycles confirmed fully reversible temperature-induced loading-release of NR.

We investigated the temperature-triggered release of doxorubicin (DOX) from **6C** micelles at 30° and 42 °C at pH = 5 to mimic tumor microenvironment (see Supporting Information for loading details). The initial 40% burst release of DOX is observed at 42 °C in 0.1 h in contrast to only 5% drug release at 30 °C (Figure 3f). After 24 h, overall 85% DOX released from the micelles at 42 °C unlike 40% of that at 30 °C. The fast drug release at 42 °C is due to the phase transition of hydrophilic micelle corona at LCST2 resulting in the disturbance of the micelle core.

In conclusion, novel temperature-sensitive block copolymers of poly(3-methyl-*N*-vinylcaprolactam) were prepared via controlled RAFT statistical copolymerization of a novel MVC monomer with VC. The new copolymer was used as the RAFT macroinitiator to prepare P(MVC-*co*-VC)-*b*-P(VC-*co*-VP) diblock copolymers with two LCSTs. The structural similarity of used monomers allowed tuning both LCSTs in the physiological range from 19 to 42 °C. The LCST1 was varied from 19 to 27 °C by copolymerization of MVC with VC and was used to form micelles loaded with a hydrophobic dye and an anticancer drug. The LCST2 at 41−42 °C was attained by the thermosensitive block obtained by RAFT copolymerization of VC with hydrophilic VP and was demonstrated useful for temperature-induced burst release of DOX. Considering their

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high loading capacity and response in physiological range, these micelles present a considerable potential as novel types of drug careers.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis, NMR and DLS characterization of copolymers, and fluorometry data for CMC determination and NR loading. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

‡These authors equally contributed to this work (X.L. and F.L.).

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Chilkoti, A.; Dreher, M. R.; Meyer, D. E.; Raucher, D. *Adv. Drug Delivery Rev.* **2002**, *54*, 613.
(2) Vaupel, P.; Kallinowski, F.; Okunieff, P. *Cancer Res.* **1989**, *49*, 6449.
(3) Fang, J. S.; Gillies, R. D.; Gatenby, R. A. *Semin. Cancer Biol.* **2008**, *18*, 330.
(4) Adair, J. H.; Parette, M. P.; Altınoglu, E. I.; Kester, M. *ACS Nano* **2010**, *4*, 4967.
(5) Larson, N.; Ghandehari, H. *Chem. Mater.* **2012**, *24*, 840.
(6) Elstrom, R. L.; Bauer, D. E.; Buzzai, M.; Karnauskas, R.; Harris, M. H.; Plas, D. R.; Zhuang, H.; Cinalli, R. M.; Alavi, A.; Rudin, C. M.; Thompson, C. B. *Cancer Res.* **2004**, *64*, 3892.
(7) Akimoto, J.; Nakayama, M.; Okano, T. *J. Controlled Release* **2014**, DOI: 10.1016/j.jconrel.2014.06.062.
(8) Lavasanifar, A.; Samuel, J.; Kwon, G. S. *Adv. Drug Delivery Rev.* **2002**, *54*, 169.
(9) (a) Riess, G. *Prog. Polym. Sci.* **2003**, *28*, 1107. (b) Rodríguez-Hernández, J.; Chécot, F.; Gnanou, Y.; Lecommandoux, S. *Prog. Polym. Sci.* **2005**, *30*, 691.
(10) Zhou, C.; Hillmyer, M. A.; Lodge, T. P. *Macromolecules* **2011**, *44*, 1635.
(11) Zhu, Z.; Sukhishvili, S. A. *ACS Nano* **2009**, *3*, 3595.
(12) Mai, Y.; Eisenberg, A. *Chem. Soc. Rev.* **2012**, *41*, 5969.
(13) Gregory, A.; Stenzel, M. H. *Prog. Polym. Sci.* **2012**, *37*, 38.
(14) Liu, F.; Urban, M. W. *Prog. Polym. Sci.* **2010**, *35*, 3.
(15) Zhang, J.; Li, X. *Prog. Polym. Sci.* **2012**, *37*, 1130.
(16) Neradovic, D.; Van Nostrum, C.; Hennik, W. *Macromolecules* **2001**, *34*, 7589.
(17) Cammas, S.; Suzuki, K.; Sone, C.; Sakurai, Y.; Kataoka, K.; Okano, T. *J. Controlled Release* **1997**, *48*, 157.
(18) Yang, C.; Attia, A. B. E.; Tan, J. P. K.; Ke, X. Y.; Gao, S. J. *Biomaterials* **2012**, *33*, 2971.
(19) Liu, J.; Debuigne, A.; Detrembleur, C.; Jérôme, C. *Adv. Healthc. Mater.* **2014**, DOI: 10.1002/adhm.201400371.
(20) (a) Vihola, H.; Laukkanen, A.; Valtola, L.; Tenhu, H.; Hirvonen, J. *Biomaterials* **2005**, *26*, 3055. (b) Liang, X.; Kozlovskaya, V.; Cox, C. P.; Wang, Y.; Saeed, M.; Kharlampieva, E. *J. Polym. Sci., Part A: Polym. Chem.* **2014**, *52*, 2725.
(21) Beija, M.; Marty, J.-D.; Destarac, M. *Chem. Commun.* **2011**, *47*, 2826.
(22) Kermagoret, A.; Fustin, C.-A.; Bourguignon, M.; Detrembleur, C.; Jerome, C.; Debuigne, A. *Polym. Chem.* **2013**, *4*, 2575.
(23) Yu, Y. C.; Li, G.; Kim, J.; Youk, J. H. *Polymer* **2013**, *54*, 6119.
(24) Liu, J.; Detrembleur, C.; De Pauw-Gillet, M.-C.; Mornet, S.; Duguet, E.; Jérôme, C. *Polym. Chem.* **2014**, *5*, 799.
(25) Hurtgen, M.; Liu, J.; Debuigne, A.; Jerome, C.; Detrembleur, C. *J. Polym. Sci., Part A: Polym. Chem.* **2012**, *50*, 400.
(26) Yu, Y. C.; Kang, H. U.; Youk, J. H. *Colloid Polym. Sci.* **2012**, *290*, 1107.
(27) Kermagoret, A.; Mathieu, K.; Thomassin, J.-M.; Fustin, C.-A.; Duchêne, R.; Jérôme, C.; Detrembleur, C.; Debuigne, A. *Polym. Chem.* **2014**, DOI: 10.1039/C4PY00852A.
(28) Tang, S. C.; Cao, Y.; Goddard, S. C.; He, W. *Polym. Chem.* **2014**, *52*, 112.
(29) Prabaharan, M.; Grailer, J. J.; Steeber, D. A.; Gong, S. *Macromol. Biosci.* **2009**, *9*, 744.
(30) Singh, P.; Srivastava, A.; Kumar, R. *Polymer* **2015**, *57*, 51.

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ACS Macro Lett. 2015, 4, 308−311