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Protein ROMP: Aqueous Graft-from Ring-Opening Metathesis Polymerization

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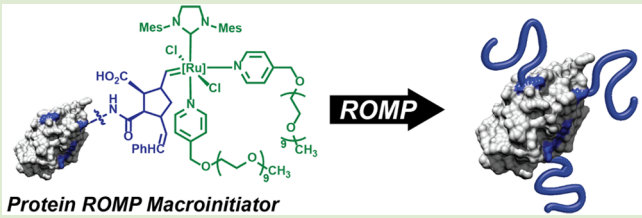
Supporting Information

ABSTRACT: Ring-opening metathesis polymerization (ROMP) was carried out from the surface of a protein under aqueous conditions. Grubbs' third generation catalyst was modified with PEGylated pyridyl groups to form a water-soluble species that showed high activity in aqueous buffered solutions at near-neutral pH. The modification of a protein with this catalyst to create a stable macroinitiator for ROMP is described. The protein macroinitiator was then used to polymerize water-soluble norbornenes, resulting in high molecular weight protein/polymer conjugates. Varying polymerization time and monomer concentration demonstrated the kinetics of molecular weight evolution and macroinitiator conversion of graft-from ROMP.



Biological therapeutics are rapidly becoming the main growth compounds in pharmaceutical development and are primarily comprised of proteins as their active components.[1] These biologics have gained interest due to their precise mechanism of action, biological specificity, and potentially reduced toxicity, as many of these compounds are already produced in the body. However, the primary problem facing protein therapeutics is the route of administration.[2] Proteins have a short half-life due to degradative enzymes in the bloodstream and therefore require repeated intravascular infusion over the course of a given treatment. To begin alleviating these concerns, a number of clinically approved therapeutic proteins are conjugated to polymers, typically poly(ethylene glycol), also referred to as PEGylation.[3,4] Polymer conjugation leads to increased circulation lifetime and decreased rates of protein degradation in vivo. Unfortunately, the chemistry necessary for the formation of these protein/polymer conjugates can be challenging. Most conjugates are formed via "graft-to" chemistry, in which a preformed polymer is coupled to a protein of interest.[4] This strategy is both kinetically and thermodynamically unfavorable as multiple macromolecules with low concentrations of reactive sites are brought together. Additionally, separating the protein/polymer conjugates from unreacted reagents is difficult since the two components are of similar size. In this work, we have pursued a "graft-from" strategy, in an effort to alleviate the aforementioned problems. The graft-from strategy involves the immobilization of an initiator directly onto the surface of a protein, yielding a protein macroinitiator from which polymers consequently grow.[5] This approach results in higher concentrations of reactive groups due to a high monomer concentration, eliminates the entropic penalty of coupling two macromolecules together, and allows for simple separation of constituent components due to the vast difference in size between the protein/polymer conjugate and the monomer units.

Several examples of graft-from bioconjugation have been reported using controlled radical polymerization (CRP) utilizing atom transfer radical polymerization (ATRP)[6,7] and reversible addition−fragmentation transfer (RAFT) polymerization chemistries.[8,9] A wide range of proteins have been grafted-from, including commercially available proteins such as lysozyme and bovine serum albumin,[8,10] as well as more advanced protein architectures, such as genetically engineered monomeric proteins[11−13] and complex protein nanoparticles.[14,15] Although this chemistry has pushed the field forward, CRP is typically limited to vinyl monomers, the polymers of which contain acrylate or acrylamide backbones. To further advance the field of protein/polymer conjugates, we have developed graft-from chemistry based on ring-opening metathesis polymerization (ROMP), which offers new sources of monomers such as norbornenes and oxanorbornenes that could be complementary to vinyl monomers.

This approach goes far beyond simply that of an alternative to traditional protein PEGylation. Norbornene monomers can be modified both pre- and postpolymerization in a variety of ways to bear useful functionalities such as imaging agents or small-molecule therapeutics, providing the ability to impart such functions to protein surfaces.[16] Together with an ability to harness the speed and fidelity of ROMP under biologically compatible conditions, this methodology has the potential to be a powerful alternative to conventional protein bioconjugation. Additionally, since the in vivo properties of poly(norbornene)-

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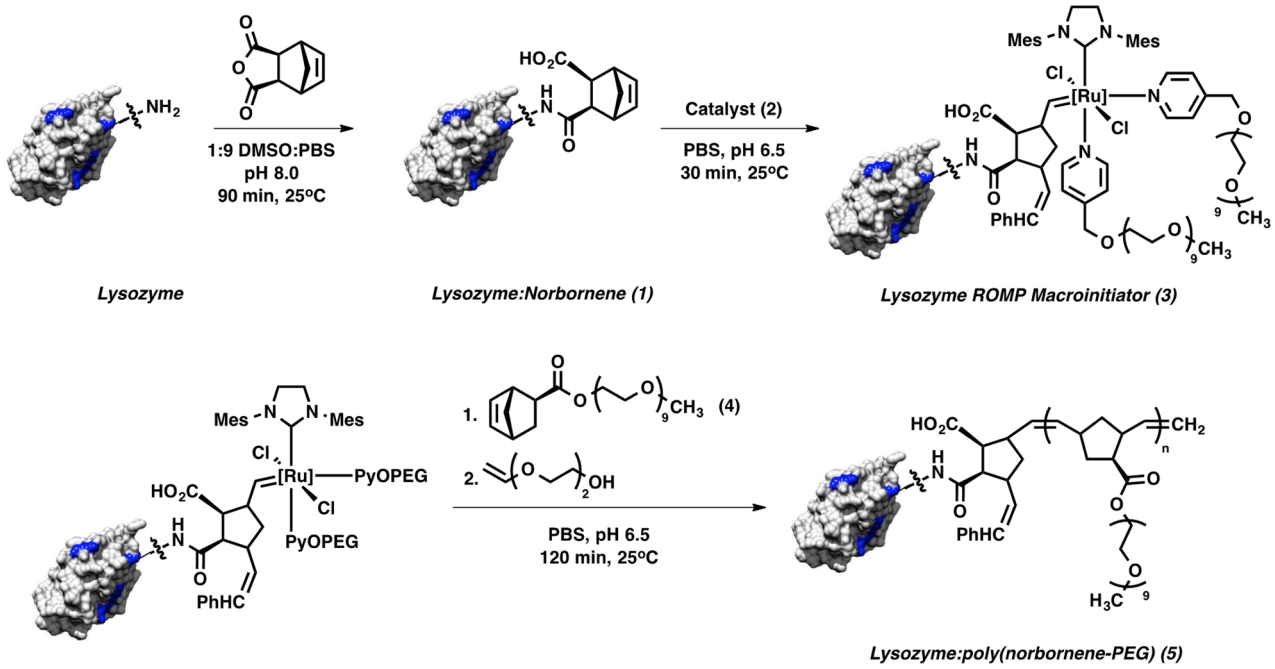
Scheme 1. Assembly of Protein Macroinitiator and Subsequent Graft-from ROMP



conjugated proteins remain unexplored, the behavior of such conjugates within biological systems must be investigated in order to determine whether the modification of proteins with functionalized polyolefins has any beneficial effects on biodistribution and pharmacokinetics.

In this report, we describe the use of ROMP as a method to prepare graft-from protein/polymer conjugates (Scheme 1). Metathesis polymerizations are extremely rapid, with many reaching completion in minutes, and are exceptionally functional group tolerant. Previous reports have shown ROMP of unprotected peptides[17] and nucleic acids,[18] as well as cross metathesis from protein surfaces.[19−21] Additionally, when polymerizing with ring-strained monomers, polymerizations are living with low dispersities and retain the ability to form block copolymers. The primary limitation of performing ROMP in aqueous media is the solubility and stability of the catalyst. A number of examples have been reported using water-soluble ruthenium-based catalysts for cross-metathesis, however, relatively few catalysts have been reported for ROMP.[22−24] In this work, we have prepared a modified water-soluble ROMP catalyst derived from the seminal work of Breitenkamp and Emrick.[25−27] A *para*-poly(ethylene glycol) substituted pyridine was prepared via Williamson ether synthesis to serve as a novel ligand for a modified Grubbs' catalyst. Ligand substitution of the pyridine groups on Grubbs' third generation catalyst resulted in water-soluble ROMP catalyst **2** (Figure 1A). ^{1}H NMR in $CDCl_3$ showed that the modified catalyst retained a strong alkylidene signal at ∼19.2 ppm, as would be expected for an active Grubbs' third generation-type species (Figure S4B). The aqueous stability of catalyst **2** was evaluated by monitoring the chemical shift and intensity of the alkylidene signal over time in D_2O. Upon dissolution in D_2O, the alkylidene peak split into multiple low-intensity peaks in the region between 18 and 19 ppm (Figure S5). This behavior is attributed to the equilibrium-exchange of the alkylidene proton for a deuterium atom and the substitution of one chlorine atom for a D_2O group, as first described by Lynn and Grubbs.[28] In addition, D_2O may partially replace one labile pyridine ligand around the ruthenium center. As a result of these effects, multiple species of catalyst **2** exist in D_2O, with each species predominantly deuterated at the alkylidene position resulting in multiple low-intensity alkylidene signals. These signals were monitored for 10 h and no significant changes in intensity or chemical shift were observed indicating that the catalyst likely retains its configuration over this period of time (Figure 1B,C). In order to evaluate the activity of catalyst **2**, ROMP of monomer **4** was performed both under organic and aqueous conditions. ROMP in CH_2Cl_2 proceeded efficiently with full monomer conversion within 30 min, as verified by ^{1}H NMR (Scheme S7). ROMP in phosphate buffer at pH 6.5 also proceeded to completion with full monomer conversion within 60 min (Scheme S8), resulting in a PEGylated poly(norbornene) with a M_n of 62.6 kDa and a PDI of 1.13 (Figure 2A,B). Previous studies have shown that the addition of copper sulfate is necessary to facilitate productive metathesis in water at near-neutral pH. Copper sulfate promotes the dissociation of pyridine ligands from the metal center, a requirement for olefin complexation and catalyst initiation.[25] However, under our experimental conditions copper additives were not necessary as ligand dissociation was likely improved as a result of the increased ionic strength of the buffered solution. The livingness of catalyst **2** was evaluated under buffered conditions. ROMP was performed with two subsequent additions of monomer **4**. After complete monomer conversion of the initial portion, as assessed via ^{1}H NMR, a second portion of monomer was added. An increase in polymer molecular weight was confirmed via GPC after the second monomer addition (Figures S10 and S11). Kinetics of catalyst **2** under aqueous conditions were evaluated by monitoring olefin signals from the monomer and polymer via ^{1}H NMR during ROMP in D_2O. The initiation rate was shown to be 2.0×10^{-3} s^{-1} (Figure 2C), falling between that of Grubbs' second and third generation catalysts in organic media, approximately 2 orders of magnitude lower than that of the bis(bromopyridine) species of Grubbs' third generation catalyst.[29]

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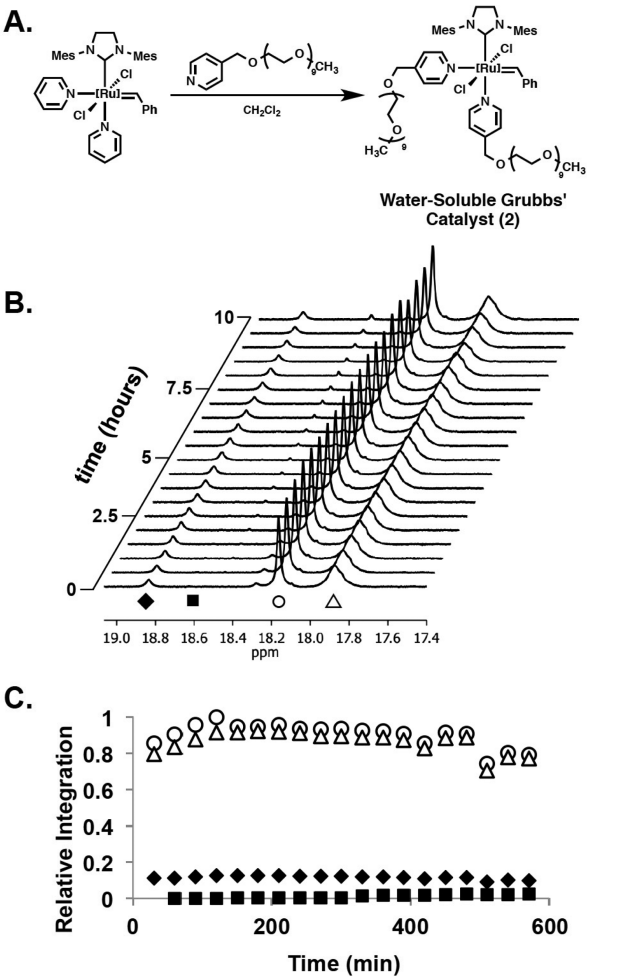

Figure 1. (A) Synthetic scheme of the modification of Grubbs' third generation catalyst to water-soluble species **2**. (B) Alkylidene region of ^{1}H NMR spectra of catalyst **2** in D_2O. Spectra were collected every 30 min for 10 h. (C) Relative integration of four alkylidene signals over 10 h.

To perform ROMP from a protein surface, the modified catalyst needed to be immobilized on a proteinaceous substrate. In this study, we used egg white lysozyme as a model protein owing to its known surface chemistry, small size, and high stability. Lysozyme was first reacted with an *exo*-norbornene dicarboxylic anhydride to modify surface-exposed lysine residues with norbornyl groups (Scheme 1). The reaction proceeded most efficiently at pH 8, yielding an average of 5−6 modifications per protein (out of 6 lysine residues), as determined by MALDI mass spectrometry (Figure S14). In principle, solvent-accessible lysine residues on the surface of any protein may be modified using this method, however care must be taken to ensure protein activity or stability if required for downstream applications. Surface modification of the protein proceeded by mixing an excess of water-soluble Grubbs' catalyst **2** with the norbornyl-modified protein **1** in PBS at pH 6.5. This afforded ring-opening of the protein-immobilized norbornene groups and insertion into the double bond, forming the protein macroinitiator **3** (Scheme 1). An excess of the catalyst was used to ensure complete and efficient attachment. Excess free catalyst was removed via centrifugal spin filtration (10 kDa MWCO). Upon multiple rounds of spin filtration, the macroinitiator was considered free from unattached Grubbs' catalyst when the filtrate became completely clear and no low-molecular weight smear from free catalyst impurity was seen via polyacrylamide gel electrophoresis (PAGE) in either the filtrate or supernatant. Since the stability of alkylidene macroinitiator **3** may differ from that of benzylidene catalyst **2**, care was taken to complete subsequent graft-from ROMP reactions within less than 2 h of macroinitiator assembly to ensure maximum catalytic activity. Graft-from polymerization proceeded by adding the macroinitiator to a solution of PEGylated norbornene monomer **4** in phosphate buffer at pH 6.5 (Scheme 1). A large excess of monomer was used to ensure initiation and the formation of conjugate. Aliquots quenched at various time points with di(ethylene glycol) vinyl ether were evaluated via PAGE to determine reaction kinetics. After 1 min, a high molecular weight protein/polymer conjugate was seen, centered at

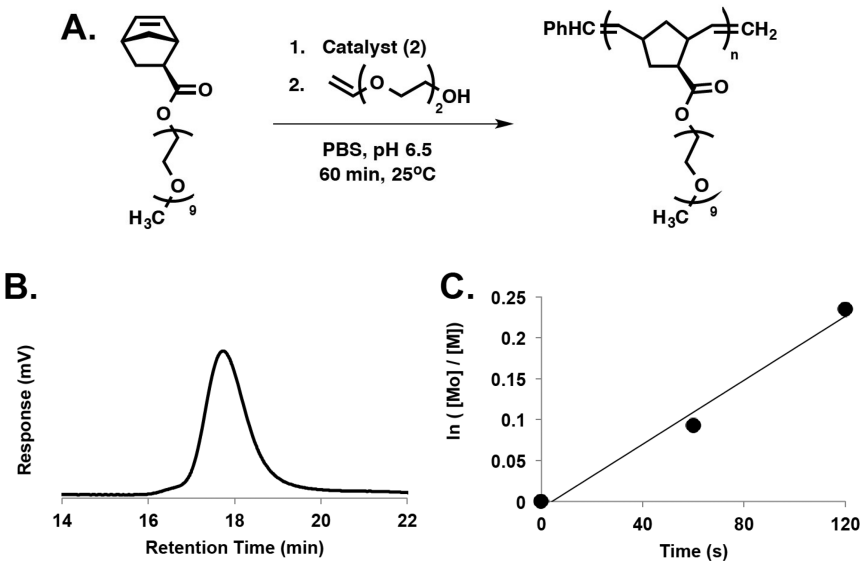

Figure 2. (A) Synthetic scheme showing ROMP of monomer **4** with catalyst **2** in phosphate buffer at pH 6.5. (B) GPC of poly(norbornene-PEG) afforded by reaction in (A). (C) Monomer conversion over the first 120 s of reaction in (A) used to determine rate of initiation of catalyst **2**.

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approximately 100 kDa (Figure 3A). After 60 min of polymerization, reactions were terminated and purified via centrifugal spin filtration (30 kDa MWCO). To evaluate the conjugation results PAGE was performed to determine approximate molecular weight. PAGE of the purified conjugate indicated a high molecular weight smear at approximately 170 kDa (Figure 3B) as has been seen in other protein/polymer conjugates synthesized via graft-from CRP polymerizations.[6,30] Encouraged by this result, the gel was stained using barium iodide, known to stain for poly(ethylene glycol) (Figure 3C).[31]

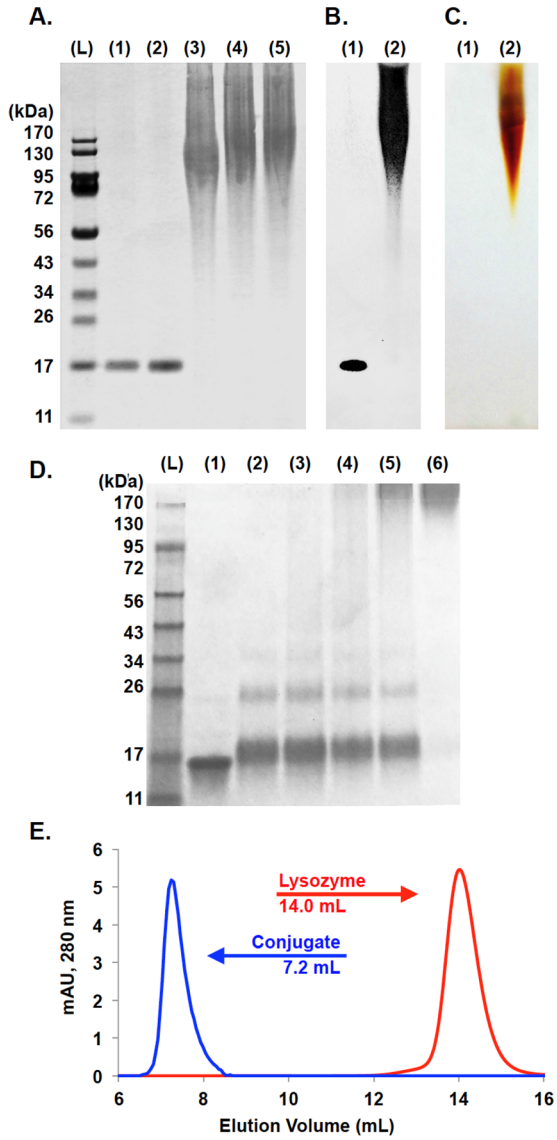

Figure 3. (A) PAGE gel of reaction mixture demonstrating kinetics; Coomassie stained. L = ladder, lane 1 = lysozyme, lane 2 = lysozyme/norbornene (**1**), lanes 3−5 = crude ROMP reactions terminated at 1, 10, and 60 min. (B) PAGE gel of purified conjugate; Coomassie stained. Lane 1 = lysozyme and lane 2 = lysozyme conjugate. (C) PAGE gel stained with barium iodide. Lane 1 = lysozyme and lane 2 = lysozyme conjugate. (D) PAGE gel of crude reaction mixture at different equivalents of monomer; Coomassie stained. L = ladder, lane 1 = lysozyme/norbornene (**1**), lane 2 = lysozyme ROMP macroinitiator (**3**), lanes 3−6 = ROMP reaction with 50, 100, 200, and 400 equiv of norbornene monomer (**4**). (E) SEC: red = lysozyme, blue = purified conjugate with 400 equiv of monomer.

The barium iodide stain matched the Coomassie stain, indicating successful protein/polymer conjugate formation. Interestingly, free poly(norbornene-PEG) also stained with Coomassie on a PAGE gel, but the pattern and color of the stain was distinctly different than that of the lysozyme/poly(norbornene-PEG) conjugate **5** (Figure S18).

Additionally, conversion percentage and molecular weight control of graft-from ROMP was evaluated by mixing equimolar aliquots of macroinitiator **3** with five monomer solutions containing 0, 50, 100, 200, and 400 equiv of monomer **4** per protein. These values correspond to theoretical polymer target weights of 0, 3.5, 7, 14, and 28 kDa per modified lysine residue. After 120 min of polymerization, the reactions were terminated, and the resulting protein/polymer conjugates were purified and characterized via PAGE and size exclusion chromatography (SEC). In this case, PAGE gels revealed that little to no ROMP initiation occurred at 50 and 100 equiv of monomer, appreciable amounts of initiation occurred at 200 equiv, and almost complete conversion occurred at 400 equiv (Figure 3D). The poor initiation in the presence of lower equivalents of monomer is likely attributed to the poor accessibility of the monomer to the active site of the docked catalyst. However, above a certain monomer concentration, the abundance of monomer forces the initiation of a catalytic cycle, becoming more kinetically favorable as the polymer chain grows and the catalyst moves further away from the protein surface. Under our experimental conditions, this critical concentration resides at ∼200 equiv of monomer per protein. PAGE of the purified conjugates showed a high molecular weight smear above ∼170 kDa. The SEC elution profile of the protein/polymer conjugates centered at ∼7 mL, within the void volume of the column. As a result, an empirical approximation of relative molecular weight via SEC was not possible. However, a significant decrease of elution volume from the elution profile of wild type lysozyme (∼14 mL), represents a large increase in molecular weight (Figure 3E).

In this report, we describe the first modification of proteins via graft-from ROMP under aqueous conditions. Growing ROMP polymers from proteins opens a new avenue toward protein/polymer conjugation. ROMP proceeds extremely rapidly and allows for a wide variety of polymer architectures. The design of norbornene monomers with a wide variety of compatible functional groups can afford a vast array of novel protein modifications. By manipulating the composition of a polymer attached to a therapeutically useful material, the potential exists to provide increased circulation time as well as for the development of novel small molecule drugs or imaging agents. In future studies, we will explore the immunogenicity and circulation lifetime of these conjugates, as well as the site-specific modification of therapeutic proteins.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details for all synthetic procedures including catalyst synthesis, protein modification, and polymerizations. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.5b00497.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Leader, B.; Baca, Q. J.; Golan, D. E. *Nat. Rev. Drug Discovery* **2008**, *7* (1), 21.
(2) Pisal, D. S.; Kosloski, M. P.; Balu-Iyer, S. V. *J. Pharm. Sci.* **2010**, *99* (6), 2557.
(3) Alconcel, S. N. S.; Baas, A. S.; Maynard, H. D. *Polym. Chem.* **2011**, *2* (7), 1442.
(4) Jevsevar, S.; Kunstelj, M.; Porekar, V. G. *Biotechnol. J.* **2010**, *5* (1), 113.
(5) Wallat, J. D.; Rose, K. A.; Pokorski, J. K. *Polym. Chem.* **2014**, *5* (5), 1545.
(6) Lele, B. S.; Murata, H.; Matyjaszewski, K.; Russell, A. J. *Biomacromolecules* **2005**, *6* (6), 3380.
(7) Bontempo, D.; Maynard, H. D. *J. Am. Chem. Soc.* **2005**, *127* (18), 6508.
(8) De, P.; Li, M.; Gondi, S. R.; Sumerlin, B. S. *J. Am. Chem. Soc.* **2008**, *130* (34), 11288.
(9) Li, M.; Li, H.; De, P.; Sumerlin, B. S. *Macromol. Rapid Commun.* **2011**, *32* (4), 354.
(10) Li, H.; Li, M.; Yu, X.; Bapat, A. P.; Sumerlin, B. S. *Polym. Chem.* **2011**, *2* (7), 1531.
(11) Gao, W.; Liu, W.; Christensen, T.; Zalutsky, M. R.; Chilkoti, A. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (38), 16432.
(12) Gao, W.; Liu, W.; Mackay, J. A.; Zalutsky, M. R.; Toone, E. J.; Chilkoti, A. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (36), 15231.
(13) Peeler, J. C.; Woodman, B. F.; Averick, S.; Miyake-Stoner, S. J.; Stokes, A. L.; Hess, K. R.; Matyjaszewski, K.; Mehl, R. A. *J. Am. Chem. Soc.* **2010**, *132* (39), 13575.
(14) Lucon, J.; Qazi, S.; Uchida, M.; Bedwell, G. J.; LaFrance, B.; Prevelige, P. E.; Douglas, T. *Nat. Chem.* **2012**, *4* (10), 781.
(15) Hovlid, M. L.; Lau, J. L.; Breitenkamp, K.; Higginson, C. J.; Laufer, B.; Manchester, M.; Finn, M. G. *ACS Nano* **2014**, *8* (8), 8003.
(16) Johnson, J. A.; Lu, Y. Y.; Burts, A. O.; Xia, Y.; Durrell, A. C.; Tirrell, D. A.; Grubbs, R. H. *Macromolecules* **2010**, *43* (24), 10326.
(17) Kammeyer, J. K.; Blum, A. P.; Adamiak, L.; Hahn, M. E.; Gianneschi, N. C. *Polym. Chem.* **2013**, *4* (14), 3929.
(18) James, C. R.; Rush, A. M.; Insley, T.; Vuković, L.; Adamiak, L.; Král, P.; Gianneschi, N. C. *J. Am. Chem. Soc.* **2014**, *136* (32), 11216.
(19) Lin, Y. A.; Chalker, J. M.; Floyd, N.; Bernardes, G. J. L.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130* (30), 9642.
(20) Lin, Y. A.; Chalker, J. M.; Davis, B. G. *J. Am. Chem. Soc.* **2010**, *132* (47), 16805.
(21) Lin, Y. A.; Boutureira, O.; Lercher, L.; Bhushan, B.; Paton, R. S.; Davis, B. G. *J. Am. Chem. Soc.* **2013**, *135* (33), 12156.
(22) Burtscher, D.; Grela, K. *Angew. Chem., Int. Ed.* **2009**, *48* (3), 442.
(23) Gallivan, J. P.; Jordan, J. P.; Grubbs, R. H. *Tetrahedron Lett.* **2005**, *46* (15), 2577.
(24) Jordan, J. P.; Grubbs, R. H. *Angew. Chem., Int. Ed.* **2007**, *46* (27), 5152.
(25) Breitenkamp, K.; Emrick, T. *J. Polym. Sci., Part A: Polym. Chem.* **2005**, *43* (22), 5715.
(26) Samanta, D.; Kratz, K.; Zhang, X.; Emrick, T. *Macromolecules* **2008**, *41* (3), 530.
(27) Dunbar, M. A.; Balof, S. L.; Roberts, A. N.; Valente, E. J.; Schanz, H.-J. *Organometallics* **2011**, *30* (2), 199.
(28) Lynn, D. M.; Grubbs, R. H. *J. Am. Chem. Soc.* **2001**, *123* (14), 3187.
(29) Love, J. A.; Morgan, J. P.; Trnka, T. M.; Grubbs, R. H. *Angew. Chem., Int. Ed.* **2002**, *41* (21), 4035.
(30) Heredia, K. L.; Bontempo, D.; Ly, T.; Byers, J. T.; Halstenberg, S.; Maynard, H. D. *J. Am. Chem. Soc.* **2005**, *127* (48), 16955.
(31) Kurfürst, M. M. *Anal. Biochem.* **1992**, *200* (2), 244.

DOI: 10.1021/acsmacrolett.5b00497
ACS Macro Lett. 2015, 4, 969−973