JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Enzyme Sensitive Synthetic Polymer Micelles Based on the Azobenzene Motif

Jingyi Rao and Anzar Khan*

Department of Materials, ETH-Zürich, CH-8093 Zürich, Switzerland

Supporting Information

ABSTRACT: In this study, we investigate the potential of an artificial structural motif, azobenzene, in the preparation of enzyme sensitive polymeric nanostructures. For this purpose, an azobenzene linkage is established at the copolymer junction of an amphiphilic diblock copolymer. This polymer assembles into a micellar structure in water. Treatment with the enzyme azoreductase, in the presence of coenzyme NADPH, results in the cleavage of the azo-based copolymer junction and disruption of the micellar assembly. These results suggest that azobenezene is a useful non-natural structural motif for the preparation of enzyme responsive polymer nanoparticles. Due to the presence of azoreductase in the human intestine, such nanomaterials are anticipated to find applicability in the arena of colon-specific delivery systems.

Synthetic micelles have been recognized as a promising class of drug delivery vehicles.[1] Therefore, developing controlled assembly/disassembly strategies in these nanostructures represents an important research goal.[2] In numerous systems, this is achieved through application of heat, light, electric field, magnetic field, or pH-based stimuli.[2,3] Biological stimuli, such as enzymes, represent an attractive alternative to meet this goal.[4] However, synthetic micellar nanostructures that respond to an enzymatic trigger remain scarce.[5] To increase the repertoire of such enzyme sensitive well-defined synthetic polymer assemblies, here, we describe a novel strategy (Figure 1). In this strategy, an azobenzene linkage is introduced, as an artificial enzyme active site, at an amphiphilic diblock copolymer junction. In water, this copolymer assembles into a micellar structure. Introduction of the enzyme azoreductase[6] to the micellar aqueous solution then triggers a disassembly process through cleavage of the azo-based copolymer linkage.[7] The significance of this system arises due to the fact that azoreductase is produced by the microbial flora present in the colon of the human intestine. Hence, azoreductase sensitive systems are useful for colon-specific delivery purposes.[8−10] This is exemplified by the azobenzene-based small molecular drug sulfasalazine that is being used in the treatment of colon diseases.[11]

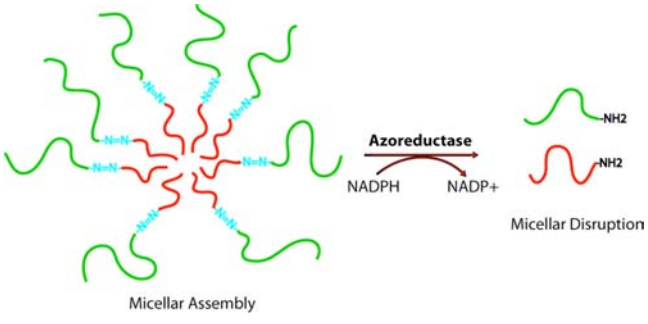


Figure 1. Schematic representation of the micellar assembly from PEG-*N*=*N*-PS block copolymer **4** and its disruption into PEG and PS homopolymers by the enzyme azoreductase in the presence of NADPH.

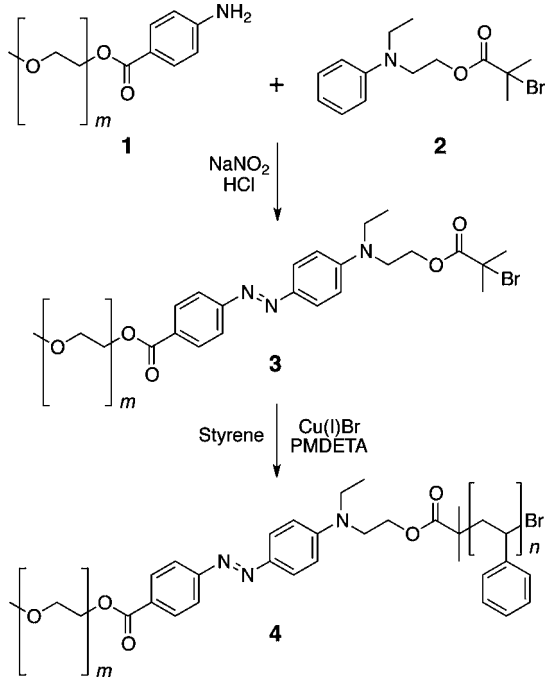
To test the aforementioned hypotheses, synthesis of the azobenzene-linked poly(ethylene glycol)-*b*-poly(styrene) (PEG-*N*=*N*-PS) amphiphilic copolymer was targeted. To accomplish this goal, aniline-end-functional PEG, **1**, was transformed into a diazonium salt and then attached to an electron-rich aromatic system, **2**.[12] Molecule **2** already contained an atom transfer radical polymerization (ATRP)[13]-based initiating site (Scheme 1). Therefore, reaction between **1** and **2** afforded PEG-based macroinitiator **3** ($M_{n(NMR)}$ = 5 kg mol^{-1}, $M_{n(GPC)}$ = 6 kg mol^{-1}, M_w/M_n = 1.11). Initiator **3** was used to polymerize styrene *via* the ATRP process. This procedure yielded diblock copolymer **4** (PEG-*b*-PS, $M_{n(NMR)}$ = 10 kg mol^{-1}, $M_{n(GPC)}$ = 18 kg mol^{-1}, M_w/M_n = 1.14) in which the two polymeric segments, PEG and PS, were connected through an azo-bond.[14,15] The ^{1}H NMR spectrum of the macroinitiator, **3**, displayed azobenzene proton resonances at 8.1, 7.9, and 6.9 ppm and PEG backbone resonances at 3.4−3.9 ppm (Figure S1). Block copolymerization with styrene resulted in the appearance of broad signals at 6.3−7.2 ppm and 1.2−2 ppm belonging to the polystyrene aromatic and aliphatic segments, respectively. Proton resonance signals from the azobenzene linker remained unchanged, indicating compatibility of the ATRP process toward the azobenzene linkage. Size exclusion chromatography (SEC) corroborated these results as block copolymerization resulted in a shifting of the elution chromatogram to a lower retention time (Figure S2).

To examine the aqueous assembly of block copolymer **4**, ^{1}H NMR spectroscopy, atomic force microscopy (AFM), transmission electron microscopy (TEM), dynamic light scattering (DLS), and UV/vis absorption spectroscopy were employed. The ^{1}H NMR spectrum of polymer **4** in deuterium oxide revealed proton resonances only from the PEG segment. This indicated that block copolymer **4** formed a micellar structure in water in which the PEG-block constituted the micellar shell and

Received: July 22, 2013
Published: September 13, 2013

dx.doi.org/10.1021/ja407514z | J. Am. Chem. Soc. 2013, 135, 14056−14059

Scheme 1. Synthesis of Azobenzene-Linked Block Copolymer 4



the PS-block constituted the micellar core domain (Figure S1).[16] AFM analysis supported this hypothesis, as spherical structures could be observed from a spin-coated sample on a silicon substrate with sizes ranging from 50 to 70 nm (Figure 2). TEM studies were then carried out on carbon coated copper grids. These studies also revealed spherical structures from an aqueous solution of block copolymer **4**. The sizes of these structures ranged from 20 to 30 nm (Figure 3). It is likely that only the electron-rich and collapsed polystyrene-based micellar core is visible under the TEM imaging. An increase in the concentration of the solution resulted in formation of string-like micellar assemblies. A further increase in the solution concentration led to the formation of larger aggregates (Figure 3b, 3c). DLS studies were then carried out to examine the secondary structure of the polymer in water (Figure S3). These studies further indicated that nanostructures with an average diameter of 65 nm were formed by the block copolymer **4** in water. Finally, UV/vis spectroscopy established a broad absorption band centered at 445 nm for the micellar solution. This absorption range is typical for the azobenzene chromophore having a donor/acceptor substitution pattern (Figure 4).[17] For this reason, the dilute aqueous micellar solution appeared yellow in color (Figure 5).

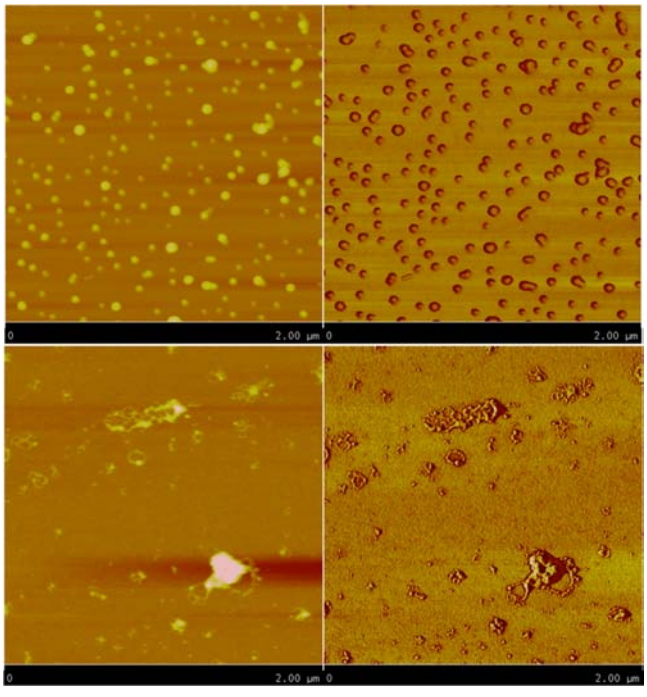


Figure 2. AFM height (left) and phase (right) images (2 × 2 μm^2) of the aqueous solution of **4** before (top) and after (bottom) the enzyme treatment.

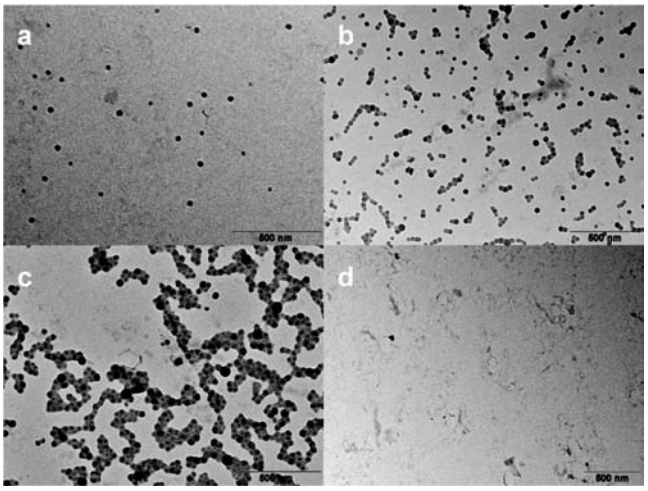


Figure 3. TEM images of the aqueous solution of **4** before (a, b, and c) and after (d) the enzyme treatment (scale bar = 500 nm).

To investigate the enzyme sensitivity of the present system, azoreductase (21 μM)[18] was introduced into the micellar aqueous solution (25 μM) along with coenzyme NADPH (65

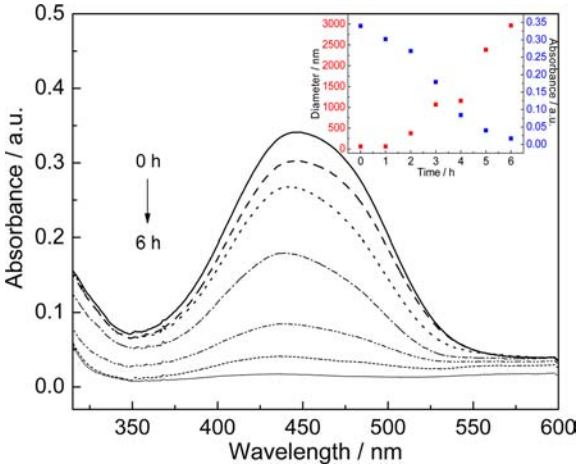


Figure 4. UV/vis absorption spectrum of aqueous solution of **4** (25 μM) at 0 (solid), 1 (dash), 2 (dot), 3 (dash dot), 4 (dash dot dot), 5 (short dash), and 6 h (short dot) after the enzyme treatment. The inset shows DLS data (red) and absorption intensity at 445 nm (blue) as a function of reaction time.

dx.doi.org/10.1021/ja407514z | J. Am. Chem. Soc. 2013, 135, 14056−14059

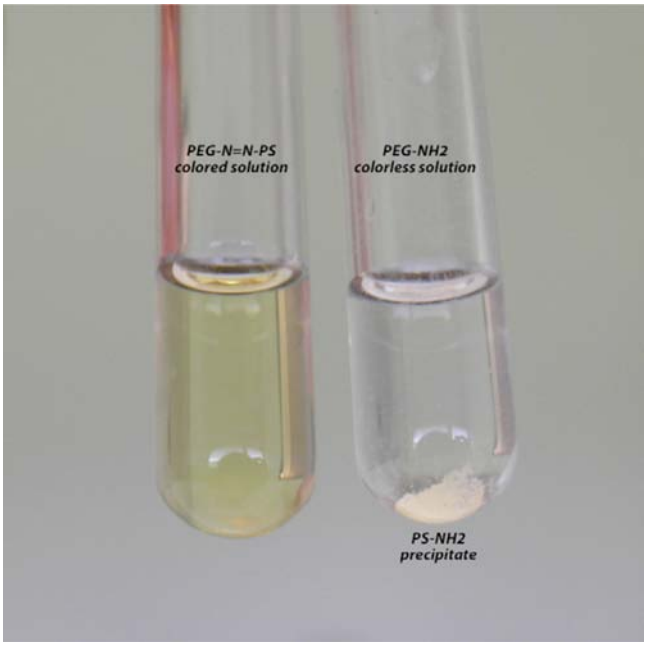


Figure 5. Digital pictures of the aqueous solution of **4** (25 μM) before (left) and after (right) the enzyme treatment.

μM) at a pH of 7 and temperature of 37 °C. The yellow polymer solution slowly turned colorless and a white solid appeared in the reaction vessel (Figure 5). This solid material was isolated through centrifugation and examined with the help of ^{1}H NMR spectroscopy. ^{1}H NMR analysis indicated that the white solid was composed of a mixture of polystyrene homopolymer and copolymer **4**. Based on area integration analysis, the ratio of the PS homopolymer to copolymer **4** was determined to be 94:6 (Figure S4). After removal of the white solid, the obtained aqueous solution was extracted with deuterated chloroform and examined with the help of ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum indicated that the aqueous solution contained the PEG homopolymer (Figure S5).

The white solid and the material obtained from the aqueous solution were then subjected to SEC analysis. As can be seen in Figure 6, the precipitate exhibited a bimodal distribution. Based on the ^{1}H NMR result (Figure S4) and comparison of the retention time, the minor peak could be assigned to copolymer **4** while the major peak could be assigned to the PS homopolymer (Figure 6). Comparison with a commercially available PS homopolymer suggested that the molecular weight of the PS generated in the enzymatic reaction was in the range of 5−6 kg mol^{-1}. This is in good agreement with the molecular weight of the PS segment (in block copolymer **4**) as calculated by end-group analysis in ^{1}H NMR spectroscopy (Figures S7 and S1). Peak deconvolution and area integration analysis in SEC suggested that the minor peak amounted to about 5−6% and the major peak to 94−95%. These numbers closely matched the quantification through ^{1}H NMR analysis. The aqueous extract, on the other hand, exhibited a similar retention time as the PEG-macroinitiator **3** (Figure 6). Along with the ^{1}H NMR result (Figure S5), this confirmed that the aqueous solution was composed of the PEG homopolymer.

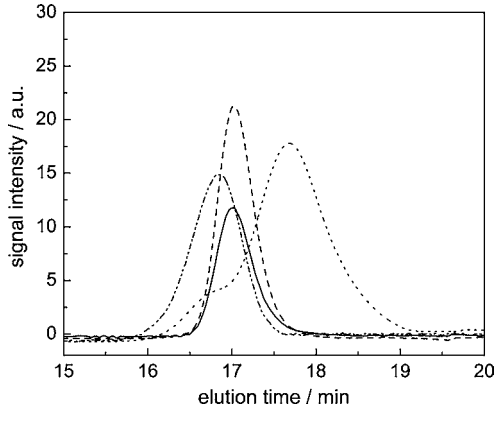


Figure 6. Size exclusion chromatograms of macroinitiator **3** (dash), diblock copolymer **4** (dash dot dot), and solid precipitate (dot) and aqueous extract (solid) obtained after the enzymatic reaction.

These results suggested that the enzymatic treatment of the micellar solution resulted in the cleavage of the azobenzene linkage placed at the junction point of the PS and PEG segments in the block copolymer **4**. Subsequently, the two polymeric segments were released from each other in solution. The PS segment precipitated out of the solution due to its insolubility in water. The PEG segment, on the other hand, remained in solution due to its miscibility in water.

AFM analysis further confirmed these conclusions as unstructured and large aggregates could be observed from the aqueous solution after treatment with the enzyme (Figure 2). These aggregates are most likely the polystyrene homopolymer suspended in water. TEM showed similar results, as the spherical nanostructures could no longer be observed after the enzyme treatment of the micellar solution (Figure 3).

UV/vis analysis indicated a continuing decrease in the absorption intensity of the azo chromophore as a function of reaction time, and no absorption signal could be located at 445 nm after 6 h of the enzymatic treatment (Figure 4). Simultaneous monitoring of the reaction mixture with the help of DLS confirmed that the decrease in the absorption intensity was directly correlated to the dissociation of the micellar nanostructures as judged by the formation of the large (micrometer sized) PS aggregates in the aqueous solution as a function of the reaction time (Figure S3 and inset of Figure 4).

To summarize, an azobenzene-linked amphiphilic diblock copolymer was prepared through an ATRP-based macroinitiator approach. This block copolymer assembled into a micellar structure in aqueous solution. Introduction of the enzyme azoreductase, in the presence of coenzyme NADPH, led to the cleavage of the azobenzene-based block copolymer linkage. This enzymatic dissociation of the copolymer connection released the two polymer segments, PEG and PS, from each other and resulted in disruption of the micellar nanostructure. The generated PEG segment remained solubilized in water, and the PS segment precipitated out of the solution. These results suggest that azobenzene is a worthy non-natural structural motif for building enzyme responsive polymer nanostructures. Due to the azoreductase sensitivity, these materials may have potential applicability in the arena of colon-specific drug delivery systems.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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

AUTHOR INFORMATION

Corresponding Author

anzar.khan@mat.ethz.ch

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Prof. Peter Walde (ETH-Z) for helpful discussions and Prof. Dieter Schlüter (ETH-Z) for constant support.

REFERENCES

(1) (a) Torchilin, V. P. *Pharm. Res.* **2007**, *24*, 1. (b) Kim, S.; Shi, Y.; Kim, J. Y.; Park, K.; Cheng, J.-X. *Expert Opin. Drug Delivery* **2010**, *7*, 49.

(2) (a) Oh, K. T.; Yin, H.; Lee, E. S.; Bae, Y. H. *J. Mater. Chem.* **2007**, *17*, 3987. (b) Spruell, J. M.; Hawker, C. J. *Chem. Sci.* **2011**, *2*, 18. (c) Wei, H.; Zhuo, R.-X.; Zhang, X.-Z. *Prog. Polym. Sci.* **2013**, *38*, 503.

(3) For selected examples, please see: (a) Gillies, E. R.; Fréchet, J. M. J. *Chem. Commun.* **2003**, 1640. (b) Gillies, E. R.; Jonsson, T. B.; Fréchet, J. M. J. *J. Am. Chem. Soc.* **2004**, *126*, 11936. (c) Goodwin, A. P.; Mynar, J. L.; Ma, Y.; Fleming, G. R.; Fréchet, J. M. J. *J. Am. Chem. Soc.* **2005**, *127*, 9952. (d) Jiang, J.; Tong, X.; Zhao, Y. *J. Am. Chem. Soc.* **2005**, *127*, 8290. (e) Lee, H.; Wu, W.; Oh, J. K.; Mueller, L.; Sherwood, G.; Peteanu, L.; Kowalewski, T.; Matyjaszewski, K. *Angew. Chem., Int. Ed.* **2007**, *46*, 2453. (f) Almutairi, A.; Guillaudeu, S. J.; Berezin, M. Y.; Achilefu, S.; Fréchet, J. M. J. *J. Am. Chem. Soc.* **2008**, *130*, 444. (g) Klaikherd, A.; Nagamani, C.; Thayumanavan, S. *J. Am. Chem. Soc.* **2009**, *131*, 4830. (h) Yan, Q.; Yuan, J.; Cai, Z.; Xin, Y.; Kang, Y.; Yin, Y. *J. Am. Chem. Soc.* **2010**, *132*, 9268.

(4) For an excellent review article on enzyme responsive materials, please see: Zelzer, M.; Todd, S. J.; Hirst, A. R.; McDonald, T. O.; Ulijn, R. V. *Biomater. Sci.* **2013**, *1*, 11.

(5) (a) Amir, R. J.; Zhong, S.; Pochan, D. J.; Hawker, C. J. *J. Am. Chem. Soc.* **2009**, *131*, 13949. (b) Azagarsamy, M. A.; Sokkalingam, P.; Thayumanavan, S. *J. Am. Chem. Soc.* **2009**, *131*, 14184. (c) Wang, C.; Chen, Q.; Wang, Z.; Zhang, X. *Angew. Chem., Int. Ed.* **2010**, *49*, 8612. (d) Ku, T.-H.; Chien, M.-P.; Thompson, M. P.; Sinkovits, R. S.; Olson, N. H.; Baker, T. S.; Gianneschi, N. C. *J. Am. Chem. Soc.* **2011**, *133*, 8392.

(6) Chen, H.; Hopper, S. L.; Cerniglia, C. E. *Microbiology* **2005**, *151*, 1433.

(7) Small molecule azobenzene derivatives are cleaved into aniline derivatives upon enzymatic action. Please refer to the following review article and references within for a detailed mechanistic picture of such enzymatic degradation: Chacko, J. T.; Subramaniam, K. *Int. J. Environ. Sci.* **2011**, *1*, 1250.

(8) For a review article, please see: Chourasia, M. K.; Jain, S. K. *J. Pharm. Pharmaceut. Sci.* **2003**, *6*, 33.

(9) Saffran, M.; Kumar, G. S.; Savariar, C.; Burnham, J. C.; Williams, F.; Neckers, D. C. *Science* **1986**, *233*, 1081.

(10) For selected examples, please see: (a) Brown, J. P.; McGarraugh, G. V.; Parkinson, T. M.; Wingard, R. E., Jr. *J. Med. Chem.* **1983**, *26*, 1300. (b) Kimura, Y.; Makita, Y.; Kumagai, T.; Yamane, H.; Kitao, T.; Sasatani, H.; Kins, I. *Polymer* **1992**, *33*, 5294. (c) Bronsted, H.; Kopecek, J. *Pharm. Res.* **1992**, *9*, 1540. (d) Mooter, G.; Samyn, C.; Kinget, R. *Pharm. Res.* **1994**, *11*, 1737. (e) Ghandehari, H.; Kopečková, P.; Kopecek, J. *Biomaterials* **1997**, *18*, 861. (f) Medina, S. H.; Chevliakov, M. V.; Tiruchinapally, G.; Durmaz, Y. Y.; Kuruvilla, S. P.; El Sayed, M. E. H. *Biomaterials* **2013**, *34*, 4655.

(11) An anti-inflammatory drug (Azulfidine in US and Salazopyrin in Europe) providing topical relief inside the intestine.

(12) He, Y.; He, W.; Wei, R.; Chena, Z.; Wang, X. *Chem. Commun.* **2012**, *48*, 1036.

(13) (a) Patten, T. E.; Xia, J.; Abernathy, T.; Matyjaszewski, K. *Science* **1996**, *272*, 866. (b) Wang, J.-S.; Matyjaszewski, K. *J. Am. Chem. Soc.* **1995**, *117*, 5614. (c) Matyjaszewski, K.; Patten, T. E.; Xia, J. *J. Am. Chem. Soc.* **1997**, *119*, 674. (d) Matyjaszewski, K.; Xia, J. *Chem. Rev.* **2001**, *101*, 2921.

(14) For approaches to cleavable polymers, please see: Rikkou, M. D.; Patrickios, C. S. *Prog. Polym. Sci.* **2011**, *36*, 1079.

(15) For macroinitiator approach to oxy-imine and hydrazone-linked amphiphilic block copolymers, please see: (a) Rao, J.; Khan, A. *Polym. Chem.* **2013**, *4*, 2691. (b) Rao, J.; De, S.; Khan, A. *Chem. Commun.* **2012**, *48*, 3427.

(16) (a) Liu, S.; Armes, S. P. *Angew. Chem., Int. Ed.* **2002**, *41*, 1413. (b) Stuparu, M. C. *Angew. Chem., Int. Ed.* **2013**, *52*, 7786.

(17) Hrozhyk, U. A.; Serak, S. V.; Tabiryan, N. V.; Hoke, L.; Steeves, D. M.; Kimball, B.; Kedziora, G. *Mol. Cryst. Liq. Cryst.* **2008**, *489*, 257.

(18) The concentration was determined using UV/vis spectroscopy (272 nm): (a) Knox, R. J.; Boland, M. P.; Friedlos, F.; Coles, B.; Southan, C.; Roberts, J. J. *Biochem. Pharmacol.* **1988**, *37*, 4671. (b) Skelly, J. V.; Sanderson, M. R.; Suter, D. A.; Baumann, U.; Read, M. A.; Gregory, D. S. J.; Bennett, M.; Hobbs, S. M.; Neidle, S. *J. Med. Chem.* **1999**, *42*, 4325.