Notes

Multifunctional Thermally Transitioning Oligopeptides Prepared by Ring-Opening Metathesis Polymerization

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Received March 6, 2007
Revised Manuscript Received May 21, 2007

Introduction

Thermally responsive polymers have been widely studied for a variety of biomedical applications including sensing, drug delivery, and as biomaterials.[1,2] These polymers exhibit lower critical solution temperature (LCST) behavior; they are soluble in aqueous solution at temperatures below their LCST and undergo a soluble-insoluble phase transition at temperatures greater than their LCST to form an insoluble, coacervate phase. Elastin-like polypeptides (ELPs) are among the most extensively studied thermoresponsive polymers for biomedical applications. ELPs are composed of amino acids (VPGXG, where X can be any amino acid except proline), are not known to elicit an immunogenic response, and further exhibit a pH-triggered phase transition.[3] The thermally or pH-triggered phase transition behaviors of ELP pentapeptide repeats can be controlled by the type and concentration of guest residue, X, molecular weight, and concentration.[4,5] Genetically engineered ELPs have been used as tags for protein purification,[6] as vehicles for drug delivery,[7,8] and as tissue engineering scaffolds in both un-cross-linked and cross-linkable forms.[9−13]

In contrast to genetically engineered ELPs, the synthesis of hybrid polymers that combine synthetic motifs with ELP repeats remains a relatively unexplored area and is the focus of this paper. In work recently reported by van Hest and co-workers,[14] low molecular weight elastin-based block copolymers were generated via atom transfer radical polymerization (ATRP) and were shown to exhibit thermally triggered phase transition behaviors as higher molecular weight, linear ELPs. This work is of interest for the potential to synthesize smaller, multifunctional, and branched elastin-based polymers and at high yield, that exhibit the attractive characteristic transitioning behaviors. The objective of the current study is to generate thermally responsive elastin-based oligopeptides of low molecular weight and low polydispersity using ring-opening metathesis polymerization (ROMP) of norbornyl elastin-based monomers. The long-term goal is to rapidly synthesize stimuli-responsive oligomers with complex functionalities or bioactive epitopes on a single molecule for a diversity of biomedical applications.

ROMP is a polymerization technique for generating materials with unique and distinct physical and chemical properties such as optical, photochromic, and electrochemical properties.[15−17] This technique has been utilized to synthesize materials for investigating multivalency in biological systems,[18−20] nanoparticles,[21,22] thin films by surface initiated ROMP chemistry,[23] and in drug delivery applications.[21] Past use of ROMP to produce oligopeptides has been limited to short peptide sequences: 4−6 amino acid residues.[24,25] The limitation in length was driven in part by the presence of amide bonds that reduce the efficiency of the catalyst. Aggregation as well as steric bulk of the monomer are also critical determinants for generating polymers with low PDIs using ROMP.[25, 26] In this study, ROMP-derived elastin-based oligomers were prepared from decapeptide monomers and characterized for their thermally responsive behaviors. The goal was to assess if ROMP was efficient for synthesis of oligomers from larger peptide monomers and to determine if the resultant elastin-based oligomers retained the characteristic inverse phase transition behavior of the larger MW, linear polypeptides. The resulting elastin-based oligomers and method for well-controlled synthesis suggests the potential to synthesize a diversity of stimuli-responsive oligomers for biomedical applications.

Experimental Section

Materials. Side chain and C-terminus protected peptide (VPGVGV-PGK(Boc)G(OtBu)) and coupling agents were purchased from Advanced ChemTech (Louisville, KY). Cell culture media and propidium iodide were purchased from Invitrogen Life Technologies (Carlsbad, CA). Viability/ Cytotoxicity Kit was purchased from Molecular Probes (Eugene, OR). Glass microscope slides and coverslips were purchased from Xenopore (Hawthorne, NJ). Dry solvents were purchased from Fisher Scientific (Springfield, NJ) and VWR International (Suwanee, GA) unless otherwise indicated. $Cl_2(PCy_3)(I_2Mes)Ru{=}CHPh$ was purchased from Fluka (Milwaukee, WI). Reactions were carried out under an Ar atmosphere in dried glassware. Moisture- and oxygen-sensitive reagents were handled in an N_2-filled drybox. 5-Norbornene-*exo*-carboxylic acid was synthesized according to the literature.[27]

Analytical thin layer chromatography (TLC) was performed on precoated silica gel plates ($60F_{254}$), and flash chromatography on silica gel-60 (230−400 mesh). TLC spots were detected by UV light and by staining with phosphomolybdic acid (PMA). Norbornyl monomer was purified by flash column chromatography on silica gel-60. The purity of oligomers was assessed by aqueous-phase size-exclusion chromatography (PL aquagel-OH 30 8 μm 300 × 7.5 mm) while simultaneously monitoring the absorption of amide bonds (220 nm) and aromatic end-group (235 and 250 nm) during elution. Inova 500 MHz NMR spectrometers were used to perform NMR analysis, and spectra were recorded in D_2O unless otherwise noted. Chemical shifts are quoted in parts per million (ppm), and ^1H NMR data are assumed to be first order. Turbidity and dynamic light scattering measurements were obtained using Cary 300 Bio Varian Instruments (Walnut Creek, CA) and DynaPro-LSR, Protein Solutions (Charlottesville, VA), respectively.

Monomer Synthesis. *General Synthesis of Norbornyl Monomer.* 5-Norbornene-*exo*-carboxylic acid was dissolved in dry CH_2Cl_2 with TBTU/HOBT (1.1 eq/0.3 eq) and DIEA (1.6 eq) followed by the addition of amine compound, VPGVGVPGK(tBoc)G(OtBu). The reaction was carried out under Ar at a final concentration of 0.08 M in the amine compound and a 1.1-fold excess of the carboxylic acid component. Upon completion of the reaction, the mixture was diluted with CH_2Cl_2 and washed with 1 N HCl and 5% $NaHCO_3$, and the organic layer was dried with Na_2SO_4. The solvent was rotary evaporated. The peptide was purified by flash chromatography eluting with 5% MeOH/CH_2Cl_2.

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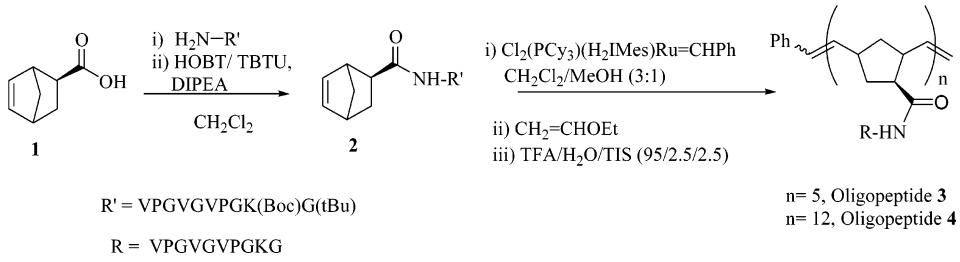
10.1021/bm0702713 CCC: $37.00
Published on Web 07/11/2007

Scheme 1. Synthesis of Elastin-Based Oligopeptide, **3** and **4** by ROMP



*Synthesis of Norbornyl-VPGVGVPGK(tBoc)G(OtBu) **2**.* VPGVG-VPGK(tBoc)G(OtBu) was coupled to 5-norbornene-*exo*-carboxylic acid to yield 240 mg (89%) of norbornyl-VPGVGVPGK(tBoc)G(OtBu), **2**. ^{1}H NMR (500 MHz): δ 9.14 (m, 1H), 8.94 (m, 1H), 8.81 (m, 1H), 8.76 (m, 1H), 8.63 (m, 1H), 8.40 (m, 1H), 7.66 (m, J = 8.5, 1H), 6.73 (m, 1H), 6.58 (m, 1H), 6.00 (m, 2H), 5.34 (m, 1H), 5.03 (m, 1H), 4.63 (m, 4H), 4.24 (m, 5H), 3.77 (m, 9H), 3.08 (m, 2H), 2.89 (s, 1H), 2.84 (s, 1H), 2.80 (s, 1H), 1.87 (m, 18H), 1.46 (s, 9H), 1.43 (s, 9H), 1.34 (m, 6H), 0.89 (m, 18H). ^{13}C (500 MHz) δ 17.99, 18.55, 19.09, 19.22, 19.27, 19.41, 19.55, 19.63, 19.86, 20.01, 22.86, 24.89, 25.15, 28.28, 28.66, 29.71, 30.26, 30.47, 32.04, 32.89, 38.82, 40.32, 41.51, 41.76, 42.12, 43.73, 43.93, 44.14, 44.34, 46.15, 48.15, 48.26, 52.98, 55.71, 56.80, 59.10, 59.58, 70.76, 79.27, 82.44, 136.20, 136.49, 137.76, 138.47, 156.26, 168.69, 168.84, 170.65, 170.74, 172.37, 172.61, 172.89, 173.60, 174.11, 175.94, 176.47. FAB, calculated for $(MH)^+$ 986.94, found 986.45

Norbornyl Oligopeptide Polymer. *Polymerization Procedure.* Catalyst $Cl_2(PCy_3)(I_2Mes)Ru{=}CHPh$ was weighed in an N_2-filled drybox and dissolved in CH_2Cl_2 to give a typical concentration of 0.03 M. Monomers were each dissolved in a minimum amount of CH_2Cl_2/CH_3OH (3/1). The desired portion of catalyst was added via syringe to the reaction bottle under an inert atmosphere. A typical reaction was carried out at an initial monomer concentration of 0.2 M. The reaction was stirred at RT for 3−4 h before quenching with ethyl vinyl ether and stirring for an additional 30 min. The solvent was concentrated by rotary evaporation and precipitated with cold Et_2O. Product was isolated by centrifugation and dried under vacuum.

Deprotection Procedure. Polymers were deprotected in a cocktail containing water/triisopropylsilane/trifluoroacetic acid (H_2O, TIS, and TFA) (2.5:2.5:95 v/v) for 4 h. The reaction mixtures were concentrated with Ar and precipitated in cold Et_2O and centrifuged. Pure deprotected product was isolated by repeated washing with Et_2O. A gray white solid was collected, dried, and stored at −20 °C. The grayish powder was redissolved in aqueous solution for turbidity and DLS measurements. The average degree of polymerization of each polymer was determined by integration of the polymer end-group (aromatic protons) versus the olefinic protons in the polymer backbone from the respective ^{1}H NMR. It should be noted that data determined via ^{1}H NMR is commonly used to predict average molecular weight of ROMP-derived polynorbornene-based oligomers.[18−20,24,25]

*Oligopeptide (**3**).* Yield 41 mg (79%) ^{1}H NMR (D_2O, 500 MHz): δ 7.20 (m), 5.20 (bs), 4.22 (m), 4.10 (m), 3.82 (m), 3.54 (m), 3.20 (s), 2.89 (m), 2.51 (m), 2.30 − 1.20 (with max. at 2.28, 1.91, 1.83, 1.52, 1.26), 0.68 (m). MALDI, calculated for $(MH)^+$ 5037.58, found 5032.74.

*Oligopeptide (**4**).* Yield 98 mg (82%) ^{1}H NMR (D_2O, 500 MHz): δ 7.23 (m), 5.19 (bs), 4.26 (m), 4.03 (m), 3.83 (m), 3.55 (m), 3.20 (s), 2.84 (m), 2.49 (m), 2.23 − 1.20 (with max. at 2.15, 1.93, 1.81, 1.54, 1.30), 0.80 (m). MALDI, calculated for $(MH)^+$ 12933.58, found 12941.99.

Primary Cell Attachment and Viability. Lumbar spines from pigs (9−12 weeks old) were obtained within 4−6 h of sacrifice (Duke University Vivarium). The intervertebral discs (IVD) were dissected under aseptic conditions and tissues harvested from the center of the disc to isolate fibrochondrocyte cells using established methods.[28] The primary cells were suspended in cell culture media (Ham's F-12 medium), supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, 0.5% fungizone, and cultured in 0.1% gelatin-coated tissue culture flasks for 2−3 days before use.

Aldehyde-functionalized glass slides were used to immobilize oligomers as follows: The glass slides were placed in a solution of the oligomer (100 μg/mL) in a neutral pH buffer for 10−15 min, and the resulting Schiff base reaction was reduced with sodium borohydride (1% solution) in water for 30 min at room temperature. The Schiff base reaction was reduced with sodium borohydride, and the slides were washed with copious water and PBS, sterilized with 70% ethanol, and washed with sterile PBS before use. Bovine serum albumin (BSA) was used for blocking all wells to inhibit nonspecific cell attachment prior to cell seeding. Cells of the intervertebral disc (5×10^5 per mL) were seeded onto the oligomer-coated surfaces, or BSA-blocked control surfaces, for 1 h before washing and reincubating in culture media. Cell nuclei were stained with propidium iodide and images taken to evaluate cell attachment at 24 h via fluorescence microscopy. Cells were also cultured out to 2 weeks and stained for ethidium homodimer and calcein AM and visualized via fluorescence and optical microscopy to evaluate cell viability and cell morphology.

Results and Discussion

The elastin-based decapeptide (VPGVGVPGK(tBoc)G(tBoc)) was coupled to 5-norbornene-*exo*-carboxylic acid (**1**) by solution-phase peptide chemistry. Compound **1** was synthesized according to the literature.[27] Both the lysine side chain and glycine C-terminus of the decapeptide were protected with acid labile protecting groups. The coupling reaction went to completion in 4 h to yield the norbornyl monomer **2** (240 mg, 89%). Compound **2** was purified by flash column then subjected to ROMP using Grubbs "second generation" ruthenium catalyst under mild reaction conditions to yield norbornyl elastin-based oligomers **3** and **4** (Scheme 1). Although the 10 amino acid-containing monomer presents a significant amount of complexity including steric bulk, the reaction was carried out in flasks submerged in an ice bath, and heating was not necessary. Residual monomers were removed during precipitation and isolation of the oligomers in cold ether.

The efficiency of the polymerization reaction was exceptionally high (>79%) despite reports that Ru catalysts are typically unstable in the presence of primary and secondary amines and

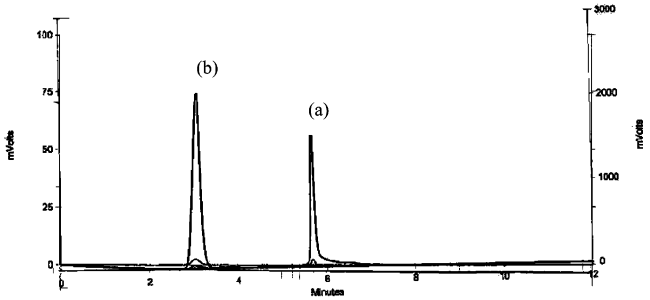


Figure 1. Overlaid SEC traces of oligomers. (a) Oligomer **3** [M]/[C] = 5, M_n = 5 kDa. (b) Oligomer **4** [M]/[C] = 12, M_n = 12 kDa. Traces are monitored at 220, 235, and 250 nm.

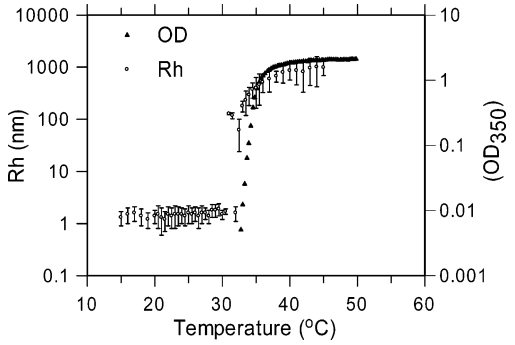


Figure 2. Particle size as a function of temperature. The hydrodynamic radii (R_h) of **3** (20 μM) was measured by DLS (mean ± polydispersity of the particle size distribution). The corresponding turbidity profile (OD) is plotted for comparison with DLS result.

Table 1. Molecular Weight and Turbidity Measurements of Norbornyl Elastin-Based Oligomers (**3** and **4**)

oligomer	$[M_o]/[C_o]^a$	avg no. (DP)^b	conc, μM	T_t (°C)
3	5/1	5	50	29.1
4	12/1	12	50	26.1

^{a} Initial feed ratio. ^{b} Determined from ^{1}H NMR.

give rise to low yields when reacting with polypeptides.[20] The apparent discrepancy might be due to the different synthetic strategy. For example, the GRGD- and PHSRN-containing oligomers prepared by Maynard and co-workers[20] were initiated in a sealed vial at 55 °C for 2 h compared to 0 °C in the current study. Even with the steric bulk of the valine at close proximity to the reaction site and several secondary amines, the reaction went to completion as observed initially by TLC. ^{1}H NMR characterization indicated the oligomers' degree of polymerization (DP) supported a 5-mer (5 kDa) and a 12 mer (12 kDa) as per our initial reaction condition: ([monomer]/[catalyst]) ratio, 5:1 and 12:1 respectively. MALDI-TOF mass spectra are consistent with the average length predicted by NMR as indicated by *m*/*z* 5032.74 and 12941.99 corresponding to oligomers **3** and **4**, respectively (see Supporting Information). Size exclusion chromatography (SEC) showed a narrow peak at 3.0 min corresponding to oligomer **4** and at 5.7 min corresponding to oligomer **3** (Figure 1). The efficiency might be in part due to the high solubility of the monomer (**2**) and the growing oligomer in the reaction solvent, and as a result, there was no need to modify the reaction condition for the synthesis of the longer oligomer (**4**). The synthesis of oligomers **3** and **4** represent the first reported preparation by ROMP chemistry of peptide-based monomers larger than six amino acids.

The thermally induced transitioning behavior of the 5 kDa oligomer was evaluated via DLS and turbidity measurements (Figure 2). The insoluble coacervates of **3** formed micron-sized aggregates that were similar in size to that described for larger molecular weight linear ELPs (50−130 kDa),[29] despite the much smaller size of the ELP oligomer (5 kDa) (Figure 2). The average hydrodynamic radius (R_h) of the particles below the transition temperature was 1.45 ± 0.07 nm (mean R_h ± polydispersity) in aqueous solution at room temperature (25 °C) for oligomer **3**. Collectively, the mean hydrodynamic radius and corresponding polydispersity, the narrow peak width obtained from SEC, and very sharp transition behaviors are trends indicative of a very narrow PDI. The elastin-based oligomer (**3**) exhibited a sharp phase transition at 34 °C at 20 μM observed on both turbidity and DLS measurements. The transition temperature (T_t) was determined by evaluating the gradient of temperature against optical density (OD) to locate the temperature of greatest change in gradient. To study the effect of molecular weight on T_t, turbidity measurements were performed on the larger oligopeptide (**4**) for comparison with **3** (Figure 3 and Table 1). The T_t of **4** was lower compared to **3** at 50 μM concentration in peptide, 26.1 and 29.1 °C, respectively. Like linear ELPs, T_t was observed to be concentration dependent, and the process was entirely reversible as seen in Figure 3. Turbidity of the oligomer solution (**3**) is lost upon cooling at a higher temperature than is generated upon heating. T_t of **3** and **4** was also concentration and pH dependent (data not shown) as well as NaCl concentration dependent (Figure 4). Varying the ionic strength between 0 and 1.5 M NaCl allowed T_t to be tuned over a 15 °C range, thus allowing control of material property isothermally. Figure 4 summarizes the effect of NaCl concentration on T_t at 20 μM in PBS for **3**. Both oligomers **3** and **4** were readily soluble in aqueous solvents without having to adjust the pH.

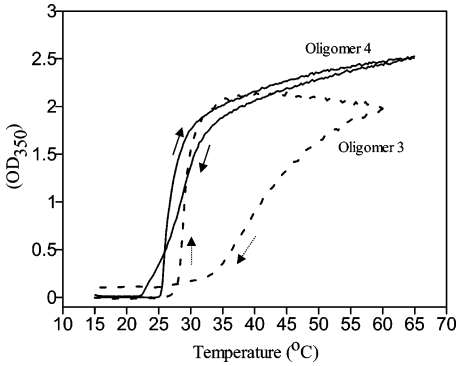


Figure 3. Turbidity profile of oligopeptide **3** and **4** (50 μM) as a function of temperature in aqueous solution. The turbidity profile was obtained heating at a rate of 1 °C/min.

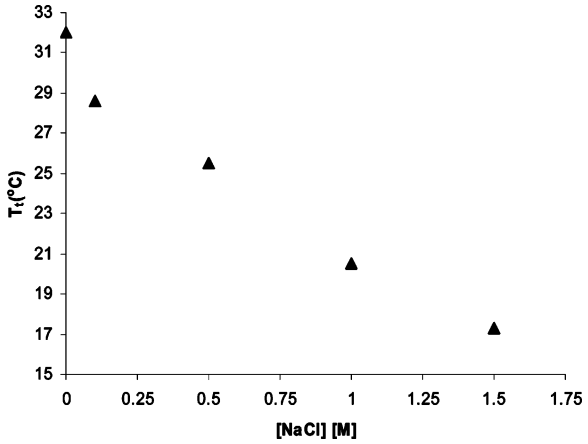


Figure 4. The effect of NaCl concentration on T_t at 20 μM oligopeptide **3** in PBS.

Fibrochondrocytes attached to slides functionalized with both oligomers **3** and **4** at an average ~8-fold greater number than for BSA-blocked blank slides (data not shown). The oligomers demonstrated an ability to support cell proliferation and the elongated and spread morphology of fibrochondrocytes in monolayer culture over 2 weeks (Figure 5, left). Virtually all cells identified at 2 weeks were found to be viable, as demonstrated by intense staining with calcein AM (Figure 5, right). It is possible that cell attachment to the oligomers is enhanced by the hydrophobic norbornyl backbone as ELPs are not known to specifically bind to cells, and as a result, biologically active sequences have been incorporated into ELP scaffolds for the promotion of attachment to endothelial cell.[30−32]

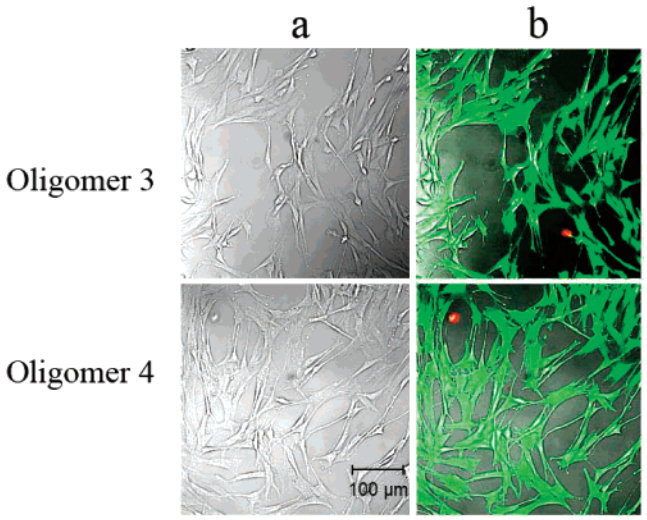


Figure 5. Phase contrast images of fibrochondrocytes isolated from intervertebral disc tissues at 15 days following attachment to surfaces coated with oligomer **3** or **4** at 100 μM (left). Images of cells obtained via fluorescence microscopy following staining with ethidium homodimer and calcein AM to assess cell viability (right). Cells were found to attach to oligomer **3** and **4** in similar numbers and to proliferate and remain viable for the 2 week culture period.

Future work will investigate the binding affinities of **3** and **4** to fibrochondrocytes as well as incorporating high affinity laminin-related peptides to increase IVD cell attachment and growth for IVD regeneration.

Conclusion

This study presents new data for ROMP-derived elastin-based oligomers of varying length that demonstrate stimuli-responsive behaviors and support cell survival and proliferation. The findings presented here demonstrate that ROMP can be performed on monomers bearing greater than six amino acid residues (10 residues) even in the presence of sterically bulky groups and several amides. Of interest was the observation that the thermal transitioning behaviors and particulate size above T_t were similar to those achieved for larger MW ELPs synthesized using recombinant protein expression. The findings for preservation of the thermally induced transition behaviors are generally consistent with findings for ATRP-generated ELP oligomers.[14] This suggests that ROMP may be used to achieve a broad range of elastin-based oligomers of short pentapeptide repeats with similar functionality to those containing long, linear polypeptide repeats. The findings that fibrochondrocytes adhere, spread, and proliferate on the oligomers suggest that the norbornene-based substrates provide no irregular cytotoxic response, illustrating the potential for a desirable biocompatibility profile. Work is also ongoing to incorporate various bioactive groups (e.g., cell adhesion peptides) into the oligomer backbone using ROMP chemistry in order to gain further multifunctionality. Additional work will focus on examinations of the stereoregularity of olefins following ROMP synthesis of the ELP oligomers and its contribution to maintaining the stimuli-responsive behaviors.

Acknowledgment. This work was supported by NIH EB00263. The authors thank Drs. Kimberly Trabbic-Carlson and Dr. Matthew Dreher for helpful suggestions. Thanks to Dr. George Dubay for help with mass spectrometry and Dr. Thangaraju Murugesan for use of the HPLC.

Supporting Information Available. Detailed procedures for the monomer and oligomer synthesis, NMR and mass spectrometry spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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BM0702713