Electronic Supplementary Material (ESI) for Chemical Communications

<Supplementary Information>

Self-assembling Nanomicelles of a Novel Camptothecin Prodrug Engineered with a Redox-responsive Release Mechanism

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Experimental Section

Materials and Methods

Poly(ethyl glycol) MW = 2,000 (PEG_{2000}) was purchased from Aladdin Chemistry, Co. (Shanghai, China). Dichloromethane (DCM) and *N, N*-dimethylformamide (DMF) were dried by refluxing over CaH_2 and distilled prior to use, whereas butanedioic anhydride, 4-dimethyl-aminopyridine (DMAP, GL Biochem, Shanghai, China), dithiothreitol (DTT, 99%, Merck, shanghai, China), triethylamine (Et_3N, 99%, Sigma, shanghai, China) and camptothecin (Zhejiang Hisun Pharmaceutical Co., Ltd, hangzhou, China) were used as received. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin, Dubelcco's phosphate buffered saline (DPBS), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Gibco Invitrogen Corp. (Shanghai, China). Paraformaldehyde solution (4% in PBS) was purchased from DingGuo Chang Sheng Biotech. Co., Ltd. (Shanghai, China). All other chemicals were obtained from Sinopharm Chemical Reagent Company (SCRC, Shanghai, China). They were of analytical grade and used as received. The dialysis bags were purchased from Spectrum Laboratories Inc. HepG2 cancer cells were kindly provided by cell center of Tumor Hospital, Fudan University (Shanghai, China).

Synthesis of CPT-COOH

CPT-COOH was synthesized according to a previously published protocol with the following modifications.[1] CPT (174 mg, 0.5 mmol) was dissolved in a mixture of 5 mM 4-dimethylaminopyridin (DMAP) (61 mg, 0.5 mmol) and 20 mM butanedioic anhydride (200 mg, 2 mmol) prepared in 100 mL of DMSO. After stirring for 24 h at 75 °C under nitrogen, following cooling of the mixture to room temperature (RT), the solution was loaded into a dialysis bag (MWCO = 3.5 kDa) and dialyzed against deionized water for 24 h. The purified intermediate (142 mg, yield 53.4%) was collected by centrifugation.

Synthesis of COOH-PEG-COOH

Oxidation of terminal PEG hydroxyl groups was accomplished following a previously published protocol with minor modifications.[2] A solution of PEG_{2000} (99%, 2.0 g, 1.0 mmol) in 15 mL of DMSO was combined at RT with 10 mL of aqueous NaOH (40 wt%) under magnetic stirring for 30 min. Chloroacetic acid (0.95 g, 10 mmol) was dropwise added and the solution continuously stirred at RT for additional 10 h. After removal of DMSO under reduced pressure, 100 mL DCM was added and precipitated salts were removed by filtration. The desired product (1.83 g, yield 85.0 %) was purified by dialysis (MWCO = 1.0 kDa) for 12 h against deionized water and lyophilized until further use.

Synthesis of H_2N-SS-PEG-SS-NH_2 [3]

A stirred solution of HOOC-PEG-COOH (1.0 g, 0.47 mmol) in DCM (25 mL) was combined with *N,N'*-dicyclohexylcarbodiimide (DCC, 116 mg, 0.564 mmol) and *N*-hydroxysuccinimide (NHS, 65 mg, 0.564 mmol) at RT under nitrogen. After 12 h, the solution was added dropwise into a round-bottom flask containing cystamine (716 mg, 4.7 mmol) dissolved in 5 mL DCM and the reaction was stirred for another 24 h. Following cooling of the mixture to 0 °C, precipitated dicyclohexylurea was removed by filtration. The filtrate was evaporated under vacuum and the residue dissolved in 10 mL of DMSO. The desired product was purified by exhaustive dialysis (MWCO = 1.0 kDa) against deionized water and collected using a membrane filter. Following suspension in 10 mL of deionized water, the intermediate H_2N-SS-PEG-SS-NH_2 (0.95 g, yield 81.0%) was lyophilized and stored at -20 °C until further use.

Synthesis of CPT-SS-PEG-SS-CPT

CPT-SS-PEG-SS-CPT was prepared following essentially the same protocol as outlined above for H_2N-SS-PEG-SS-NH_2. Briefly, a stirred solution of CPT-COOH (300 mg, 0.70 mmol) in DMSO (150 mL) was combined with DCC (173 mg, 0.84 mmol) and NHS (96.6 mg, 0.84 mmol) at RT under nitrogen. After 12 h, a solution of H_2N-SS-PEG-SS-NH_2 (560 mg, 0.23 mmol) in 10 mL of DMSO was added dropwise and the reaction stirred for 24 h. Following solvent removal under reduced pressure,

the residue was dissolved in DMSO (10 mL), combined with 10 mL of deionized water, and transferred into a dialysis bag (MWCO = 3.5 kDa) for purification. After removing the precipitation by using a membrane filter, CPT prodrug (325 mg, yield 41.0%) was collected by lyophilizing the filtrate.

Characterization Methodologies

Proton nuclear magnetic resonance (^{1}HNMR) spectra were recorded on a Bruker AV 500 NMR spectrometer (Bruker, German). In general, samples were prepared at 5 mg/mL in DMSO-*d6*. UV spectra were recorded using a Cary 50 UV-Vis spectrophotometer (Varian, Ltd., Hong Kong, China), whereas fluorescence spectra were acquired on a Hitachi F-2500 fluorescence spectrophotometer (Hitachi, Ltd, Hong Kong, China). Molecular weights of CPT-SS-PEG-SS-CPT and PEG_{2000} were estimated by GPC using Agilent HP1100 system equipped with a G1314A pump and a G1362A differential refractive index detector (Agilent, USA). Separations were performed at 35 °C using tetrahydrofuran (THF) as the eluent (flow rate = 1.0 mL/min). The size distribution of CPT-SS-PEG-SS-CPT micellar aggregates was determined by DLS using Nano-ZS 90 Nanosizer (Malvern Instruments Ltd., Worcestershire, UK). Morphology of CPT-SS-PEG-SS-CPT micellar aggregates was assessed by TEM using a JEOL JSM-200CX transmission electron microscope (Hitachi, Ltd., Hong Kong, China) at an acceleration voltage of 100 kV. Routinely, a drop of nanomicelle suspension containing 0.01% (w/v) phosphotungstic acid was placed on a copper grid with formvar film and dried before measurement.

Chromatographic studies of CPT-SS-PEG-SS-CPT

Chromatographic studies were performed on Agilent 1100 HPLCs (Agilent, Palo Alto, CA, USA), equipped with an autosampler, thermostat-column device, a variable-wavelength UV detector operating at 250 nm and a data acquisition system using the HP Chemstation software. The analysis

was achieved on Kromasil C18 column (150 mm×4.6 mm, packed with 3.5 μm diameter particles, Agilent Technologies, MN, USA) using Methanol-water (85:15, v/v) as a mobile phase with the flow-rate 1.0 ml/min at 29 ^{o}C. Injection volume was 10 μl.

Determination of CPT Loading Efficiency

The content of CPT in CPT-SS-PEG-SS-CPT was quantified by UV/Vis spectroscopy based on the standard curve of CPT at λ = 365 nm. CPT loading efficiency was defined by the amount of CPT conjugated to PEG versus the weight of CPT-SS-PEG-SS-CPT.

Nanomicelle Formation

Aqueous suspensions of CPT-SS-PEG-SS-CPT nanomicelles were prepared by dialysis at RT. Briefly, CPT prodrug (2 mg) was dissolved in DMF at an initial concentration of 0.5 mg/mL and dialyzed for 24 h against 2.0 L of deionized water (MWCO = 3.5 kDa). The water was changed every 12 h.

CMC Determination

CMC of CPT-SS-PEG-SS-CPT was determined by fluorescence spectroscopy using pyrene as hydrophobic florescence probe that preferentially partitions into the micelle core causing changes in photophysical properties of the nanoparticles under investigation.[5] Aliquots of pyrene solutions (6 μM in acetone, 100 μL) were added to glass containers, and acetone was allowed to evaporate at 40 ^{o}C. Subsequently, 2 mL of aqueous prodrug solutions at concentrations ranging from 2.4×10^{-4} to 0.5 mg/mL were then added. The solutions were kept at room temperature for 24 h to allow establishment of solubilization equilibrium of pyrene in the aqueous phase. Fluorescence spectra were recorded at an excitation wavelength of λ = 310 nm, while fluorescence emission was collected at λ = 350, 378, and 397 nm, respectively. The CMC was estimated by extrapolating the cross-point in fluorescence intensity from experimental values.

Stability of CPT-SS-PEG-SS-CPT Nanomicelles

Micellar aggregates of CPT-SS-PEG-SS-CPT (0.5 mg/mL) prepared in 150 mL of PBS, pH 7.4 were incubated with 10 mM of the water-soluble reducing agents DTT at 37 °C. Time-dependent changes in nanomicelle size distribution were monitored for 24 h by DLS. Control experiments without DTT were performed in parallel.

CPT Release from Prodrug Nanomicelles

Release kinetics of CPT from micellar aggregates of the novel CPT-SS-PEG-SS-SS-CPT prodrug were measured at 37 °C following a modified protocol by Koo etal.[4] Routinely, 5 mL of a CPT-SS-PEG-SS-SS-CPT nanomicelle suspension prepared in PBS, pH 7.4 at 0.5 mg/mL were filled into a dialysis bag (MWCO = 3.5 kDa in the presence and absence of 10 mM DTT. Dialysis was performed against of deionized water (150 mL) under constant stirring (150 rpm). Over a period of 240 h, 2 mL aliquots of the dialysis medium were withdrawn at predefined time points and quantified for CPT by UV/Vis spectroscopy at λ = 365 nm. Experiments were performed in triplicate, and results are reported as mean ± SD.

Cytotoxicity of CPT Prodrug Nanomicelles

HepG2 cells were maintained in DMEM supplemented with 10% (v/v) FBS and 1% (w/v) of penicillin/streptomycin. For experiments, cells were dissociated from plastic support using trypsin and seeded into 96-well flat-bottomed tissue-culture plates at a density of 5,000 cells/well. Cells were allowed to attach overnight in a humidified atmosphere of 5% (v/v) CO_2 at 37 °C before exposed to 100 µL of CPT prodrug nanomicelles diluted in culture medium to 5 - 640 µg/mL in the presence and absence of 10 mM GSH. After a 24 h incubation, 200 µL of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution prepared in DMEM (5 mg/mL) was added to each well. Subsequently, cells were incubated for additional 4 h at 37 °C allowing viable cells to reduce the MTT into purple formazan crystals. 100 µL of DMSO were added to each well to dissolve formazan crystals before absorbance was measured at λ = 492 nm using a Multiscan MK3 plate reader (Thermo Fisher

Scientific, Waltham, MA, USA). The relative cell viability in % was calculated according to: cell viability = ($OD_{treated}/OD_{control}$) × 100%, where $OD_{treated}$ represents the absorbance of treated cells after subtraction of absorbance of control wells containing only cell culture medium.

Cellular Uptake of CPT Prodrug Nanomicelles

HepG2 cells routinely maintained in DMEM supplemented with 10% (v/v) FBS and 1% (w/v) of penicillin/streptomycin were seeded in a 6-well plate at a density of 1×10^5 cells/well. Cells were allowed to attach overnight in a humidified atmosphere of 5% (v/v) CO_2 at 37 °C. Before experiments, cells were washed with prewarmed PBS, pH 7.4 and incubated with 100 μg/mL of CPT-SS-PEG-SS-SS-CPT nanomicelles at 37 °C using complete culture medium. After a 24 h incubation period, cells were washed twice with PBS, pH 7.4 and fixed with 4% (w/v) formaldehyde in PBS. The slides were mounted for confocal laser scanning microscopy (Olympus, FV300, IX71, Tokyo, Japan), and intracellular CPT was detected at an excitation wavelength of $\lambda = 405$ nm.

Statistical Analysis

The group comparisons were made by analysis of variance. Data are presented as means ± standard errors.

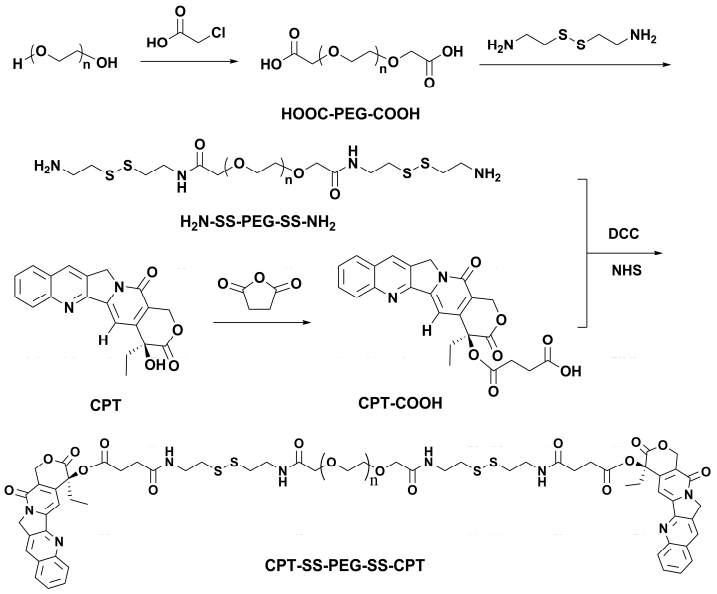

Figure S1. Synthetic strategy used to prepare CPT-SS-PEG-SS-CPT copolymer with redox-sensitive disulfide linker.

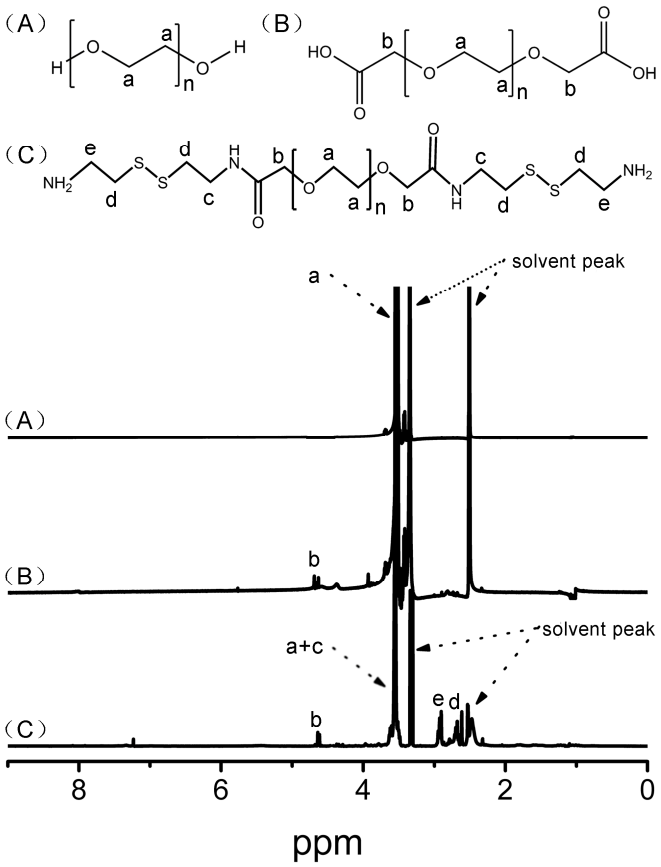

Figure S2. ^{1}H NMR spectra of (A) PEG_{2000}, (B) HOOC-PEG-COOH, (C) H_2N-SS-PEG-SS-NH_2 in DMSO-*d*6.

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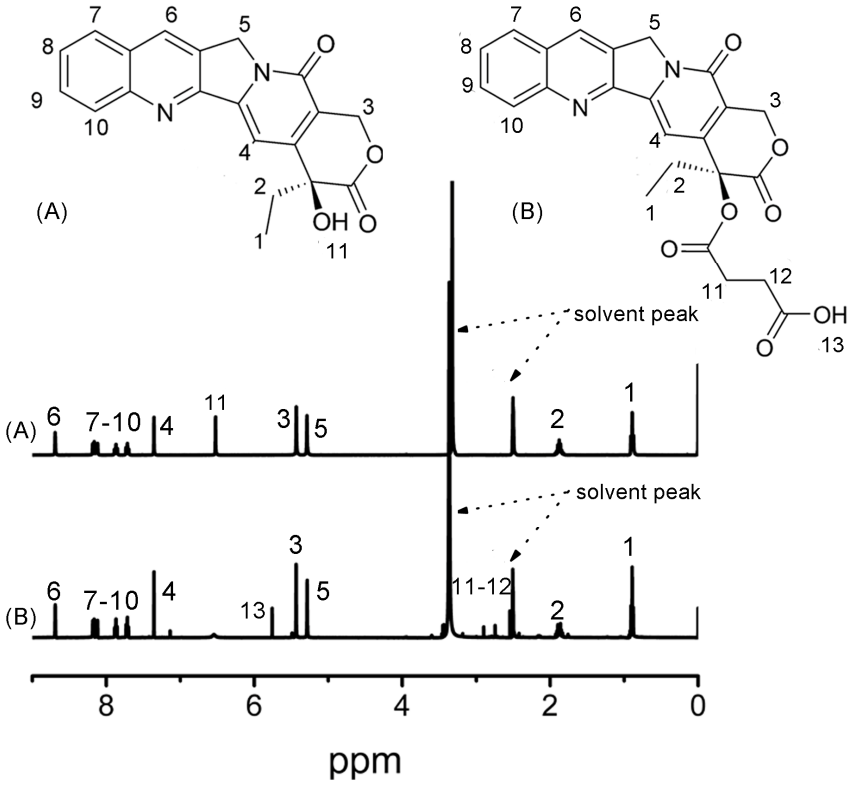

Figure S3. ^{1}H NMR spectra of (A) CPT and (B) CPT-COOH in DMSO-*d*6.

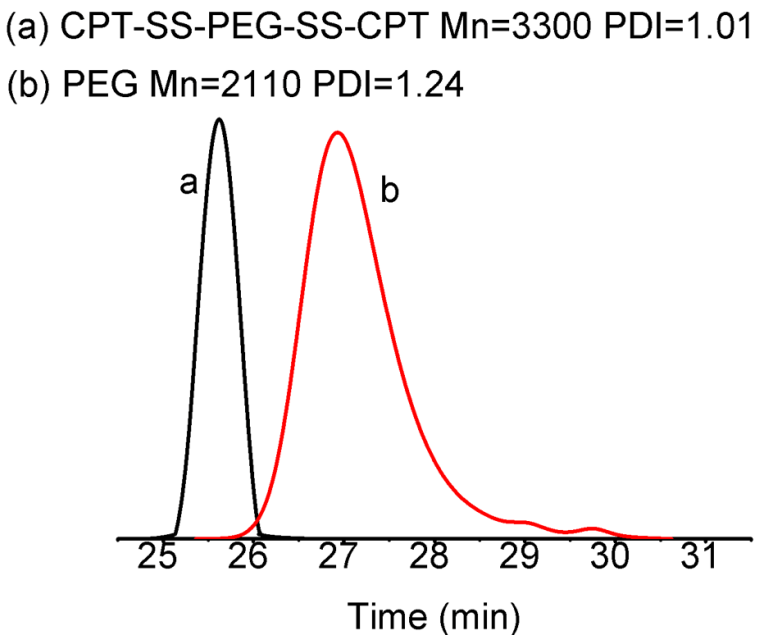

Figure *S4*. GPC elution profiles of PEG_{2000} and synthesized CPT-SS-PEG-SS-CPT prodrug.

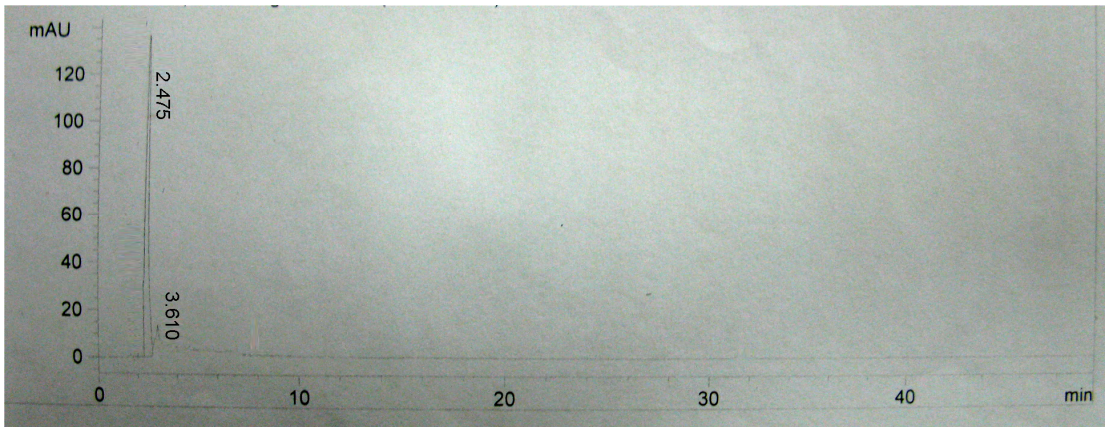

Figure *S5*. Analytical HPLC profile for CPT-SS-PEG-SS-CPT (UV detection at 250 nm).

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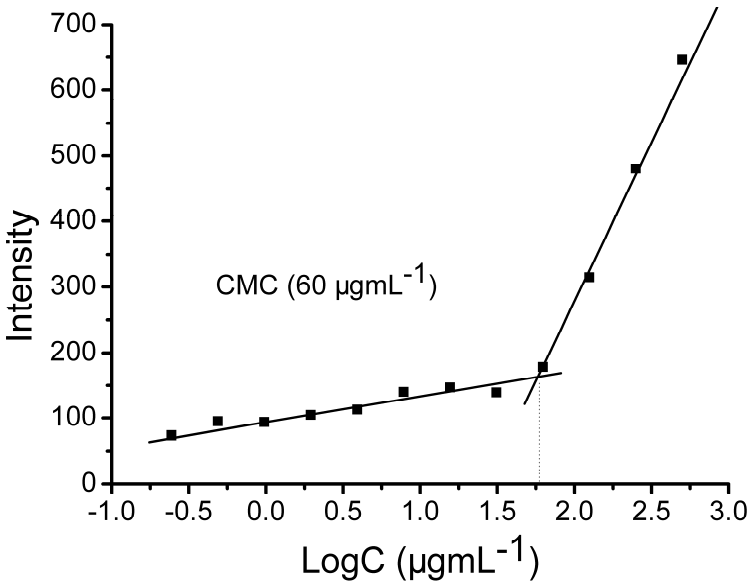

Figure *S6*. The fluorescence intensity of pyrene at 350 nm under excitation as a function of the logarithm of polymer concentration.

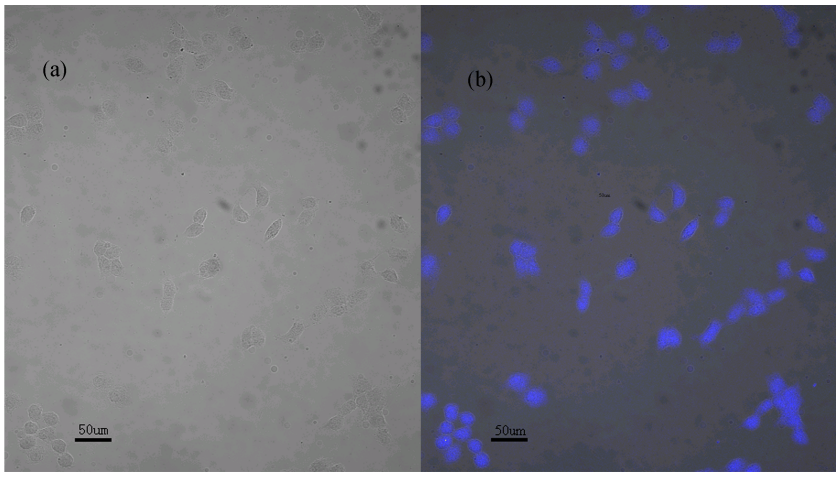

Figure S7. Confocal laser scanning micrographs of HepG-2 cells after a 24 h incubation with CPT-SS-PEG-SS-CPT prodrug nanomicelles (a) bright field view, (b) fluorescence view with blue for CPT.

References

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5. J. W. Bae, E. Lee, K. M. Park and K. D. Park, *Macromolecules.* 2009, **42,** 3437-3442.