

Article

Dual Location, Dual Acidic pH/Reduction-Responsive Degradable Block Copolymer: Synthesis and Investigation of Ketal Linkage Instability under ATRP Conditions

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Supporting Information



ABSTRACT: Stimuli-responsive degradation (SRD) undergoing chemical transition through the cleavage of labile linkages has been proved to dramatically increase the versatility of stimuli-responsive block copolymers. In particular, dual or multiple stimuliresponsive degradable block copolymers that can be triggered by two endogenous stimuli of acidic pH and reduction are in high demand. Here, a new strategy utilizing atom transfer radical polymerization (ATRP) is reported to synthesize a dual acidic pH/ reduction-responsive degradable block copolymer (DLDSRD) labeled with an acidic pH-labile ketal linkage at the block junction and pendant reductively cleavable disulfide groups in hydrophobic block at dual locations. A robust route with multiple steps utilizing carbamate chemistry to endow stability during protection/deprotection steps enables the synthesis of a novel poly(ethylene glycol)-based ATRP macroinitiator labeled with a ketal linkage (PEG-ketal-Br macroinitiator). Conducting ATRP allows for the synthesis of a series of DLDSRD diblock copolymers consisting of a hydrophilic poly(ethylene glycol) block covalently conjugated through a ketal linkage with a hydrophobic polymethacrylate block having multiple disulfide pendants. Analysis shows an unexpectedly high degree of polymerization of the hydrophobic polymethacrylate block that could be attributed to the instability of ketal linkages under ATRP conditions. The preliminary results from aqueous micellization and dual acidic pH/reduction-responsive cleavage of ketal and disulfide linkages suggest the feasibility of DLDSRD-based nanoassemblies toward effective drug delivery exhibiting precisely controlled release in response to dual stimuli at dual locations (core and interfaces).

INTRODUCTION

In recent years stimuli-responsive (or smart) copolymers and particularly block copolymers have emerged as promising building blocks of choice in the construction of advanced nanomaterials for the plethora of applications in nanoscience, nanotechnology, and pharmaceutical science.¹⁻⁷ Upon being triggered by external stimuli, these stimuli-responsive copolymers undergo either a physical or a chemical transition, depending on the nature of stimuli-responsive moieties (or groups) within the structures. In comparison with a physical transition leading to a change in volume or phase in response to physical stimuli (mostly, temperature and pH),⁸⁻¹⁰ chemical transition involves the incorporation of cleavable (or labile) covalent bonds into the design of block copolymers. So-called stimuli-responsive degradable copolymers degrade to their appropriate products upon the cleavage of labile linkages when chemical or biological stimuli are present.¹¹⁻¹³ As a consequence, SRD polymers, particularly those with amphiphilic properties (called SRD-exhibiting amphiphilic block copolymers), and their self-assembled nanoassemblies have been specifically studied for on-demand drug delivery.¹⁴⁻¹⁶ Acidic pH and light as well as reductive, oxidative, and enzymatic reactions are typical stimuli of great interests and promises that can cleave the corresponding labile linkages. In particular, acidic pH has received an increasing attention owning to the slightly acidic pH of tumor microenvironment (pH = 6.5-6.9) as well as endosomes and lysosomes (pH = 5-6) as compared to normal tissues (pH = 7.4).¹⁷ Acetal, ketal, orthoester, hydrazone, imine,

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and β -thiopropionate are typical acidic pH-labile linkages.^{18,19} In addition to acidic pH, reductive reaction has been extensively explored with unique disulfide linkage as a promising reduction-responsive degradable linkage.^{20–22} In biological environments, cellular glutathione (a tripeptide having cysteine) is present at millimolar concentrations (\approx 10 mM) in an intracellular compartment, compared with that (<10 μ M) in extracellular compartment, and further elevated concentration (3–4 times) in tumor tissues and cancer cells.²³

Given numerous strategies to synthesize novel SRD block copolymers labeled with a single type of cleavable linkage exhibiting single stimulus response,^{24–26} increasing attention has been drawn to stimuli-responsive block copolymers that can be triggered by two or more stimuli.^{27–29} Such dual or multistimuli responsiveness displays analogous features to natural macromolecules within the body, whose behavior is governed by cumulative effects of stimuli rather than a single factor. Various strategies that have enabled the synthesis of dual- and multistimuli-responsive block copolymers have been reviewed.³⁰⁻³² Most strategies have utilized a combination of chemical, biological, and physical stimuli.³³⁻⁴⁴ Despite these advances, the development of SRD block copolymers having only stimuli-responsive cleavable (degradable) linkages is more promising because of the propensity to complete and control disassembly or chemical degradation of nanoassemblies.⁴ Furthermore, dual and multiple stimuli-responsive degradable (MSRD) block copolymers which can be triggered by two endogenous acidic pH and reduction are in high demand.⁴

A few examples of dual acidic pH/reduction-responsive degradable (or cleavable) block copolymers have been demonstrated, where acidic pH-cleavable linkages are positioned in the side chains of hydrophobic blocks^{50,51} or as crosslinks, 52-55 thus single location. Other block copolymers were also synthesized with acidic pH-labile acetal or benzoic imine linkage positioned at the junction of hydrophilic and hydrophobic blocks. However, they were brush-type block copolymers⁵⁶⁻⁵⁹ and linear polycaprolactone-based triblock copolymers⁶⁰ with a single disulfide linkage positioned in the center of the main chains. To the best of our knowledge, no reports describe a dual acidic pH/reduction-responsive degradable block copolymer comprising a ketal group at the block junction and disulfide pendants in the hydrophobic blocks. The copolymer self-assembles to nanoassemblies with multiple disulfide linkages in the cores and ketal linkage at the core/corona interfaces, thus attaining dual location, dual acidic pH/disulfide-responsive degradation. Such a strategy can offer the versatility in that multistimuli responses to each stimulus can independently and precisely regulate the release of encapsulated biomolecules at dual or multiple locations.^{61,62} Compared with acetals and other acid-labile linkages, $^{63-65}$ the ketal group is highly reactive to the cleavage in environment, particularly acidic pH, thus leading to rapid hydrolysis rate and unique degradation pattern.⁶⁶ However, its degradation can be varied with substituents as well as their hydrophobic-hydrophilic environments in the copolymers where they are situated.⁶⁷

In this work, we have investigated a new strategy utilizing atom transfer radical polymerization (ATRP) to synthesize a dual location, dual acidic pH/reduction-responsive degradable block copolymer (DLDSRD). The diblock copolymer is composed of a hydrophilic PEG block covalently conjugated through a ketal linkage with a hydrophobic polymethacrylate block having pendant disulfide linkages (PHMssEt), thus PEG-ketal-PHMssEt block copolymer. The strategy requires the synthesis of a novel PEG-based bromine macroinitiator labeled with a ketal linkage (PEG-ketal-Br) for the ATRP of HMssEt. A robust route with multiple steps utilizing carbamate chemistry stable to hydrolytic conditions and protection/deprotection chemistry is allowed for the synthesis of the macroinitiator. Other routes were also investigated to understand the unexpected side reactions associated with the high sensitivity of ketal linkage. A series of ATRP of HMssEt in the presence of the synthesized PEG-ketal-Br as well as the instability of ketal group under ATRP conditions were systematically investigated. Further, the synthesized block copolymer after purification was characterized for aqueous micellization through self-assembly and dual acidic pH/ reduction-responsive cleavage of ketal and disulfide linkages.

EXPERIMENTAL SECTION

Instrumentation. ¹H NMR spectra were recorded using a 500 MHz Varian spectrometer. The $CDCl_3$ singlet at 7.26 ppm and DMSO- d_6 quintet at 2.5 ppm were selected as the reference standard. Spectral features are tabulated in the following order: chemical shift (ppm); multiplicity (s = singlet, d = doublet, t = triplet, m = complex multiple); number of protons; position of protons. Monomer conversion was determined using ¹H NMR in CDCl₃. Molecular weight and molecular weight distribution were determined by gel permeation chromatography (GPC). An Agilent GPC was equipped with a 1260 Infinity isocratic pump and a RI detector. Two Agilent PLgel mixed-C and mixed-D columns were used with DMF containing 0.1 mol % LiBr at 50 °C at a flow rate of 1.0 mL/min. Linear poly(methyl methacrylate) standards from Fluka were used for calibration. Aliquots of the polymer samples were dissolved in DMF/LiBr. The clear solutions were filtered using a 0.40 μ m PTFE filter to remove any DMF-insoluble species. A drop of anisole was added as a flow rate marker. The size of micelles in hydrodynamic diameter by volume was measured by dynamic light scattering (DLS) at a fixed scattering angle of 175° at 25 °C with a Malvern Instruments Nano S ZEN1600 equipped with a 633 nm He-Ne gas laser.

Materials. Most reagents including triethylamine (Et₃N), bromoisobutyryl bromide (Br-iBuBr), and tin(II) 2-ethylhexanoate (SnEH₂, 95%) used in our synthesis were purchased from Sigma-Aldrich Canada and used as received, except for disuccinimidyl carbonate (DSC) from Toronto Research Chemicals and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide-HCl salt (EDC) from Matrix Innovation. Poly-(ethylene glycol) monomethyl ether (PEG, MW = 5000 g/mol, EO# = 113) was dried by azeotropic distillation with toluene to remove residual moistures. Solvents include ethyl acetate (EA), hexane (HE), dichloromethane (DCM), tetrahydrofuran (THF), and methanol (MeOH). Tris(2-pyridylmethyl)amine (TPMA),⁷⁰ a methacrylate having a pendant disulfide linkage (HMssEt),⁷¹ and PEG-functionalized bromide (PEG-Br)⁷² were synthesized as described elsewhere.

Synthesis of PEG-Ketal-Br Initiator. The detailed procedures for the synthesis of PEG-ketal-Br are described here. Other attempts to synthesize the initiator are detailed in the Supporting Information.

AC1. Ethyl trifluoroacetate (50.2 g, 354 mmol) was added dropwise to a solution consisting of ethanolamine (18.0 g, 295 mmol) and Et₃N (44.7 g, 442 mmol) dissolved in MeOH (300 mL) in an ice bath at 0 °C for 20 min, and then the mixture was stirred at room temperature for 12 h. After the removal of solvents by rotary evaporation, the residues were dissolved in saturated brine (100 mL), extracted from EA (200 mL) four times, and dried over sodium sulfate. After solvent was evaporated, the product was purified by silica gel column chromatography with EA as an eluent. The product, a white solid, was collected as the first of a total two bands from a silica gel column; yield 35.5 g (76.5%). $R_f = 0.5$ on silica (EA as an eluent). ¹H NMR (CDCl₃, ppm): 6.85 (s, 1H, F₃CC(O) <u>NHC</u>-), 3.80 (t, 2H, F₃CC(O)NH<u>CH₂CH₂OH</u>), 3.54 (t, 2H, F₃CC(O)NHCH₂<u>CH₂OH</u>), 2.05 (s, 1H, F₃CC(O)NHCH₂<u>CH₂OH</u>). ¹³C NMR (DMSO, ppm): 158.0, 116.03, 59.45, 42.10. Mass calculated for (C₄O₂NF₃H₆Na⁺): 180.02428. Found: 180.02451.

AC2. **AC1** (12.2 g, 77.6 mmol) dissolved in anhydrous THF (300 mL) was mixed with pyridinium *p*-toluenesulfonate (PPTS) (1.2 g, 4.6

mmol) under vigorous stirring for 1 h. After the addition of molecular sieves (5 Å, 1.6 mm pellet, predried at 100 °C for 72 h, 150 g), the resulting mixture was kept at 0 °C for 30 min under magnetic stirring. Followed by the addition of a solution of 2-methoxypropene (1.4 g, 19.4 mmol) dissolved in cold anhydrous THF (15 mL), the mixture was stirred overnight at room temperature and then quenched by the addition of Et₃N (6.2 mL). Molecular sieves by filtration and solvents by rotary evaporation were removed; then residues were dissolved in phosphate buffered saline (PBS, pH 7.4, 150 mL) and extracted from EA (200 mL) three times. After solvent was evaporated, the product was purified by silica gel column chromatography (1/1 v/v EA/HE). The product, a white solid, was collected as the second of the total three bands off a silica gel column, yielding 3.3 g (48.2%). $R_f = 0.67$ on silica (1/1 v/v EA/HE). ¹H NMR (CDCl₂, ppm): 6.8 (s, 2H, CF₃C(O) NHCH2CH2OC(CH3)2O-), 3.5 (s, 8H, F3CC(O)NHCH2CH2OC-(CH₃)₂O-), 1.37 (s, 6H, -CH₂OC(<u>CH₃</u>)₂OCH₂-). ¹³C NMR (CDCl₃, ppm): 158.0, 116.03, 101.0, 59.45, 39.92, 22.5. Mass calculated for (C₁₁O₄F₆N₂H₁₆Na⁺): 377.09065. Found: 377.09190.

AC3. AC2 (2.8 g, 7.9 mmol) was dissolved in 6 M aqueous NaOH solution (60 mL) and stirred for 4.5 h at room temperature. The product was extracted from DCM (150 mL) and dried over sodium sulfate. Solvent was evaporated by rotary evaporation to yield an amber-colored oil (1.2 g, 89.7%). ¹H NMR (CDCl₃, ppm): 3.4 (t, 4H, NH₂CH₂-CH₂OC(CH₃)₂O-), 2.8 (t, 4H, NH₂CH₂CH₂OC(CH₃)₂O-), 1.37 (s, 6H, $-CH_2OC(CH_3)_2OCH_2-$). ¹³C NMR (CDCl₃, ppm): 100.50, 63.23, 42.2, 25.12. Mass calculated for (C₇N₂O₂H₁₈Na⁺): 185.12605. Found: 185.12578.

AC4. Ethyl trifluoroacetate (0.87 g, 6.2 mmol) was dropwise added to a clear solution containing AC3 (1.0 g, 6.2 mmol), Et₃N (0.74 g, 7.4 mmol), and MeOH (15 mL) in an ice bath for 20 min. The reaction mixture was stirred overnight at room temperature, and then solvents were evaporated. The residues were dissolved in PBS (pH 7.4, 50 mL) and extracted from DCM (100 mL) three times. After the evaporation of solvent, the product was purified by silica gel column chromatography deactivated with Et₃N using DCM/MeOH (9/1 v/v). The product, a yellow oil, was collected as the second of the total three bands off a silica gel column, yielding 0.35 g (22.4%). $R_{f} = 0.54$ on silica (8/2 v/v DCM/ MeOH; ninhydrin was used for visualization). ¹H NMR (CDCl₃, ppm): 3.68 (t, 2H, -CF₃C(O)NH<u>CH₂CH₂OC(CH₃)₂O-)</u>, 3.54 (m, 4H, $-\underline{CH}_2OC(CH_3)_2O\underline{CH}_2-$), 2.8 (t, 2H, $-OC(CH_3)_2OCH_2\underline{CH}_2NH_2)$, 1.37 (s, 6H, $-CH_2OC(CH_3)_2OCH_2-$). ¹³C NMR (CDCl₃, ppm): 157.5, 116.0, 99.9, 61.65, 58.05, 40.95, 40.45, 24.74. Mass calculated for (C₉N₂O₃F₃H₁₇Na⁺): 281.10830. Found: 281.10770.

PEG-DSC. A solution containing PEG (5.0 g, 1.0 mmol), Et₃N (0.30 g, 3.0 mmol), and DSC (0.77 g, 3.0 mmol) dissolved in anhydrous DCM (220 mL) was purged with nitrogen for 20 min and then stirred for 28 h at room temperature. The product, a white solid, was precipitated from anhydrous diethyl ether (600 mL), isolated by vacuum filtration, and dried in a vacuum oven at room temperature for 12 h, yielding 5.0 g (97%). ¹H NMR (CDCl₃, ppm): 4.45 (t, 2H, <u>CH₂OC(O)-</u>), 3.45–3.80 (m, -<u>CH₂CH₂O- of PEG main chain</u>), 3.37 (s, 3H, <u>CH₃O-</u>), 2.83 (s, 4H, -(O)C<u>CH₂CH₂C(O)-</u>).

AC5. A solution containing the purified, dried PEG-DSC (1.5 g, 0.29 mmol) and Et₃N (0.06 g, 0.61 mmol) in chloroform (110 mL) was mixed with a solution of **AC4** (0.17 g, 0.67 mmol) in chloroform (5 mL) in an ice bath. The mixture was stirred for 22 h at room temperature. The product, a white solid, was precipitated from anhydrous diethyl ether (500 mL), isolated by vacuum filtration, and dried in a vacuum oven at room temperature for 12 h, yielding 1.4 g (93.2%). ¹H NMR (CDCl₃, ppm): 4.21 (t, 2H, <u>CH₂OC(O)-</u>), 3.45–3.80 (m, <u>-CH₂CH₂O- of PEG main chain), 3.37 (s, 3H, <u>CH₃O-</u>), 1.36 (s, 6H, <u>-CH₂OC-(CH₃)₂OCH₂-).</u></u>

 $A\bar{C}6$. A solution of ACS (1.4 g, 0.26 mmol) dissolved in MeOH (30 mL) was mixed with a solution of potassium carbonate (0.25 g, 1.85 mmol) in water (6 mL). The mixture was stirred overnight at room temperature and concentrated by rotary evaporation. The residues were dissolved in water (50 mL) and extracted from DCM (250 mL) three times. The product, a white solid, was precipitated from anhydrous diethyl ether (500 mL), isolated by vacuum filtration, and dried in a vacuum oven at room temperature for 12 h, yielding 1.23 g (91.7%). ¹H

NMR (CDCl₃, ppm): 4.21 (t, 2H, <u>CH₂OC(O)</u>–), 3.45–3.80 (m, $-\underline{CH_2CH_2O}$ of PEG main chain), 3.37 (s, 3H, <u>CH₃O</u>–), 2.85 (t, 2H, – OCH₂<u>CH₂NH₂</u>), 1.36 (s, 6H, $-CH_2OC(\underline{CH_3})_2OCH_2$ –).

PEG-Ketal-Br (AC7). Br-iBuBr (0.13 g, 0.59 mmol) was added to a solution containing AC6 (1.23 g, 0.24 mmol) and Et₃N (0.35 g, 3.56 mmol) dissolved anhydrous THF (100 mL) in an ice bath. The mixture was stirred for 90 min. The formed solids (Et₃N-HBr adducts) were removed by vacuum filtration, and solvent was evaporated. The residues were dissolved in DCM (300 mL), and the solution was washed with water two times and then dried over sodium sulfate. The product, a white solid, was precipitated from anhydrous diethyl ether (500 mL), isolated by vacuum filtration, and dried in a vacuum oven at room temperature for 12 h, yielding 1.1 g (87.9%). ¹H NMR (CDCl₃, ppm): 4.21 (t, 2H, <u>CH₂OC(O)–</u>), 3.45–3.80 (m, –<u>CH₂CH₂O– of PEG main chain</u>), 3.37 (s, 3H, <u>CH₃O–</u>), 1.95 (s, 6H, –NHC(O)C(<u>CH₃)₂Br</u>)), 1.36 (s, 6H, –CH₂OC(CH₃)₂OCH₂–).

Synthesis of DLDSRD Block Copolymers by ATRP. To synthesize DLDSRD-1 as an example, PEG-ketal-Br (0.24 g, 45.7 μ mol), HMssEt (0.8 g, 2.28 mmol), [Cu(II)TPMABr]Br (1.17 mg, 2.27 μ mol), TPMA (1.99 mg, 6.8 μ mol), and anisole (3.5 g) were mixed in a 15 mL Schlenk flask. The mixture was deoxygenated by purging under nitrogen for 1 h and then placed in an oil bath at 40 °C. A nitrogen prepurged solution of Sn(II)(EH)₂ (7.41 mg, 18.3 μ mol) dissolved in anisole (0.5 g) was injected into the Schlenk flask to initiate polymerization. Polymerization was stopped after 2.3 h by cooling the reaction mixture in an ice bath and exposing it to air. For kinetic studies, aliquots of the samples were taken periodically to follow monomer conversion using ¹H NMR analysis. Polymerization was stopped by cooling and exposing the reaction mixture to air.

A similar procedure was applied except for the use of PEG-ketal-Br (0.13 g, 25.6 μ mol), HMssEt (0.45 g, 1.28 mmol), [Cu(II)TPMABr]Br (0.66 mg, 1.28 μ mol), TPMA (1.12 mg, 3.85 μ mol), and anisole (3.0 g) for DLDSRD-2 and PEG-ketal-Br (0.18 g, 33.7 μ mol), HMssEt (0.3 g, 0.85 mmol), [Cu(II)TPMABr]Br (0.88 mg, 1.71 μ mol), TPMA (1.49 mg, 5.13 μ mol), and anisole (3.0 g) for DLDSRD-3.

For purification, the as-prepared polymer solution was diluted with acetone and passed through a basic alumina column to remove residual copper species. The solvent was removed under rotary evaporation at room temperature; the polymer was isolated by precipitation from hexane and then dried under vacuum at room temperature for 15 h.

ATRP To Synthesize PEG-*b*-PHMssEt Block Copolymer (Control). PEG-Br (0.10 g, 19.4 μ mol), HMssEt (0.34 g, 0.97 mmol), [Cu(II)TPMABr]Br (0.5 mg, 0.97 μ mol), TPMA (0.85 mg, 2.92 μ mol), and anisole (3.4 g) were mixed in a 15 mL Schlenk flask. The mixture was deoxygenated by purging under nitrogen for 1 h and then placed in an oil bath at 40 °C. A nitrogen prepurged solution of Sn(II)(EH)₂ (3.15 mg, 7.77 μ mol) dissolved in anisole (0.5 g) was injected into the Schlenk flask to initiate polymerization. Polymerization was stopped after 2 h by cooling the reaction mixture in an ice bath and exposing it to air.

¹H NMR Investigation of Ketal Cleavage under ATRP Conditions. PEG-ketal-Br (30 mg, 5.6 μ mol) dissolved in DMSO- d_6 (1 mL) was mixed with CuBr₂ (2 mg, 8.95 μ mol). ¹H NMR spectra of the resulting mixture were recorded over time.

Dual Stimuli-Responsive Cleavage of DLDSRDs. Selected with DLDSRD-1, for acidic pH response, its aliquot (1 mg) dissolved in $CDCl_3$ was mixed with a drop of HCl. After 45 min, the mixture was analyzed by ¹H NMR. For dual responses, its aliquot (10 mg) dissolved in DMF (10 mL) was mixed with DTT (4 mg, 1.2 mol equiv to pendant disulfides) and a drop of HCl (0.63 mmol) under stirring at room temperature for 1 day. An aliquot was taken to analyze molecular weight distribution using GPC.

Aqueous Micellization Using the Dialysis Method. Water (10 mL) was added dropwise to an organic solution of DLDSRD-1 (15 mg) in THF (2 mL) using a syringe pump equipped with a plastic syringe (20 mL volume, 20 mm diameter) at an addition rate of 0.2 mL/min. The resulting dispersion was placed in a dialysis tubing with MWCO = 3500 g/mol and dialyzed against water (1 L) for 24 h. Outer water was changed twice to yield aqueous micellar aggregates at 1.5 mg/mL.

Macromolecules

RESULTS AND DISCUSSION

Synthesis of PEG-Ketal-Br ATRP Initiator. Our approach to synthesize PEG-ketal-Br initiator began with the synthesis of a diamine precursor labeled with a ketal linkage (AC3) as described in the previous publications.⁷³ As illustrated in Scheme 1, the first step was the protection of amine group in ethanol

Scheme 1. Synthetic Route to AC3



amine with ethyl trifluoroacetate in the presence of Et_3N (a base) in MeOH to synthesize AC1 at 76% yield. The second step was the reaction of AC1 with 2-methoxypropene in the presence of pyridinium *p*-toluenesulfonate (PPTS) in THF, yielding AC2 at 48% yield. The use of molecular sieves with 5 Å pore size is essential to remove the formed MeOH. Both AC1 and AC2 were purified by column chromatography, and their strucutures were confirmed by ¹H NMR and ¹³C NMR analysis (Figures S1 and S2), along with high-resolution mass spectroscopy to determine their absolute molecular weights. The next step was the deprotection of trifluoroacetamide groups in AC2 in the presence of 6 M aqueous NaOH solution. ¹H and ¹³C NMR (Figures 1a and S3) as well as further COSY NMR analysis (Figure S4) confirm the synthesis of AC3 at 89% yield.



Figure 1. ¹H NMR spectra of **AC3** (a) and **AC4** (b) in CDCl₃. Note that the values under each spectrum are integrals.

Given the successful synthesis of AC3, Scheme 2 illustrates our successful route (I) to the synthesis of PEG-ketal-Br functionalized with a ketal linkage and a bromo group (AC7). This synthetic route centers on the use of carbamate linkage between PEG and AC4, which is stable under alkaline deprotection processes. A few reports describe the utilization of stable carbamate linkages for the synthesis of polymer–drug conjugates and hydrogels.^{74–76} The first step was the protection of one amine group in **AC3** with ethyl trifluoroacetate (an electronwithdrawing group) in a basic condition. Column chromatography was required to isolate **AC4** from corresponding dimer at 22% yield. The chemical structure of the purified **AC4** was confirmed by ¹H NMR (Figure 1b) and ¹³C NMR (Figure S5) as well as further COSY NMR analysis (Figure S6).

In a separate set, the hydroxyl group of PEG was activated with DSC in the presence of Et₃N. After purification from diethyl ether, the structure of the formed PEG-DSC is confirmed by ¹H NMR analysis (Figure 2a). The second step was the conjugation of PEG-DSC with AC4 through the formation of carbamate bond to synthesize AC5. The third step was the treatment of AC5 with K₂CO₃ in a mixture of MeOH and water. This step allows for the cleavage of trifluoroacetamide group to generate the corresponding amino group, thus yielding AC6. Both AC5 and AC6 were purified by precipitation from diethyl ether, and the purified products were characterized by ¹H NMR analysis (Figure 2b,c). For example with AC6, the typical peaks include the peak (b) corresponding methylene group adjacent to ester bond, the peak (f) to two methyl groups in the ketal linkage, and the peak (h) to methylene group adjacent to amine group. Their integral ratio was close to 2/6/2, which is equivalent to that of their proton numbers. Further, the complete deprotection of trifluoroacetamide protecting group is confirmed with ¹⁹F NMR spectroscopy (Figure S7). The final step was the coupling reaction of the purified AC6 with Br-iBuBr to synthesize AC7. HBr (a strong acid) is released as a result of the coupling reaction. Thus, the use of excess Et_3N (15 mol equiv to AC6) to facilitate the formation of HBr-Et₃N salts as well as the reaction time of <3 h at room temperature were adopted, in order to minimize the unexpected cleavage of ketal linkages during coupling reaction. The ¹H NMR spectrum in Figure 2d shows the peak (j) corresponding to two methyl groups in bromo moieties. The quantitative mole ratio of the peaks a/f/j = 3/6/6, suggesting the successful synthesis of PEG-ketal-Br.

Given the robust route (I) that allows for the successful synthesis of PEG-ketal-Br, two other routes (II and III), which were not straightforward and successful, were also explored in an attempt to synthesize PEG-ketal-Br. As illustrated in Scheme S1, the route (II) is similar to the above route (I), but differs with the use of ester bond between PEG and AC4. The route (III) in Scheme S2 centers on the synthesis of AC12 functionalized with both amine and bromo groups, which could yield a PEG-ketal-Br by its direct coupling with a COOH-functionalized PEG (PEG-COOH). The detailed procedures and results including NMR spectra (Figures S8–S13) are described in the Supporting Information.

Synthesis of PEG-Ketal-PHMssEt and Kinetic Investigation. Given our successful synthesis and characterization of PEG-ketal-Br initiator, atom transfer radical polymerization (ATRP), a successful control radical polymerization (CRP), was examined to synthesize PEG-ketal-PHMssEt (DLDSRD) block copolymers. The Activators ReGenerated by Electron Transfer (ARGET) process for ATRP^{77,78} was employed since this process requires the use of a minimal amount of Cu species (<50 ppm). As illustrated in Figure 3, the polymerization was mediated with Cu(II)/TPMA complexes in the presence of the PEG-ketal-Br initiator. Sn(II)EH₂ was used as a reducing agent to convert Cu(II) species to active Cu(I) species. Under the conditions for typical ARGET ATRP including [PEG-ketal-Br]₀/[Cu(II)Br₂]₀/ [TPMA]₀/[Sn(II)EH₂]₀ = 1/0.05/0.15/0.4 in anisole at 40 °C, Scheme 2. Synthetic Route (I) to PEG-Ketal-Br Initiator, Starting with AC3



Figure 2. ¹H NMR spectra of PEG-DSC (a), AC5 (b), AC6 (c), and AC7 (d, PEG-ketal-Br) in $CDCl_3$. Note that the values under each spectrum are integrals. x denotes impurities including water.

the initial mole ratio of $[HMssEt]_0/[PEG-ketal-Br]_0$ as the targeting degree of polymerization (DP) of PHMssEt at the complete monomer conversion was varied with 50/1 and 25/1. Polymerization was stopped and ¹H NMR analysis was used to determine conversion.

After purification by filtration through a basic alumina column to remove residual metal species and following precipitation from hexane to remove unreactive monomers, the copolymers were characterized for their structures and molecular weights. For an example with DLDSRD-1, its ¹H NMR spectrum in Figure 3 shows the typical peaks at 3.7 ppm corresponding to methylene protons in PEG block and 0.8–1.2 ppm equivalent to methyl protons on backbones in PHMssEt block. Their integral ratio was analyzed to determine the DP of PHMssEt to be 164 at conversion = 0.66. GPC analysis in Figure 4 (red line for DLDSRD-1) indicates the molecular weight as the number-



10⁵

10⁴

PEG-ketal-Br M_n = 12.7 kg/mol

M_w/M_n = 1.02

10³

average molecular weight $(M_n) = 87.4$ kg/mol with molecular weight distribution as $M_w/M_n = 1.18$. Note that a small peak observed in high molecular weight region could be attributed to high molecular species formed by undesired side reactions during

10⁶

<u>DLDSRD-3</u> M_n = 55.6 kg/ M_w/M_n = 1.11

<u>DLDSRD-1</u> M_n = 87.4 kg/ M_w/M_n = 1.18

DLDSRD	$[HMssEt]_0/[I]_0$	HMssEt/anisole (w/w)	time (h)	conv ^b	DP ^c PHMssEt	$M_{\rm n}^{\ d} ({\rm kg/mol})$	$M_{\rm w}/M_{\rm n}^{\ d}$
1	50	0.23	2.3	0.66	164	87.4	1.18
2	50	0.10	6.4	0.58	159	51.4	1.07
3	25	0.15	2.0	0.54	110	55.6	1.11

Table 1. Characteristics and Properties of DLDSRD Block Copolymers Synthesized by ARGET ATRP of HMssEt in the Presence of PEG-Ketal-Br^a

^{*a*}Conditions for ATRP: $[PEG-ketal-Br]_0/[Cu(II)Br_2]_0/[TPMA]_0/[Sn(II)EH_2]_0 = 1/0.05/0.15/0.4$ in anisole at 40 °C. ^{*b*}Determined by ¹H NMR. ^{*c*}Calculated by conversion × target DP at 100% conversion. ^{*d*}Determined by GPC with PMMA standards.

ATRP. Similar protocols were used to analyze other DLDSRD copolymers, and their characteristics and results are summarized in Table 1.

Kinetics of ATRP of HMssEt in the presence of PEG-ketal-Br was investigated by analyzing samples taken periodically (not purified). Figure 5 shows that conversion linearly increased with



Figure 5. First-order kinetic plot over polymerization time for ARGET ATRP of HMssEt in the presence of PEG-ketal-Br in anisole at 40 °C.

time, suggesting that the polymerization is first-order. This result indicates the constant concentration of active centers during polymerization, up to 60% conversion. The polymerization was well-controlled. As expected, polymerization slowed down when the wt ratio of HMssEt/anisole decreased (more anisole) from 0.23 (DLDSRD-1) to 0.10 (DLDSRD-2) at 40 $^{\circ}$ C.

Instability of Ketal Linkages during ATRP. Our careful analysis suggests that all three copolymers after purification presented in Table 1 had the DPs of PHMssEt blocks much greater than those calculated based on conversion and targeting DPs. For example with the purified DLDSRD-3, the DP of PHMssEt block was 110 determined by ¹H NMR analysis, while it can be estimated to be 14, based on conversion (0.54) and targeting DP = 25. The plausible reason for such large discripency of the DPs of DLDSRD block copolymers were investigated.

The samples taken during ATRP to investigate its kinetics were analyzed for the DPs of PHMssEt block. Note that those samples were not purified by our standard protocol (filtration with basic Al_2O_3 column and then precipitation from hexane). Our ¹H NMR analysis indicates that the determined DPs of the samples were very close to DPs theoretically calculated with targeting DP over conversion (Figure 6). This result appeared to be quite different from the DPs (110–164) of the purified DLDSRD copolymers. We then carefully examined the purification steps of the copolymers.



Figure 6. DP determined by ¹H NMR analysis for samples taken during ATRP over conversion. The theoretical DP values are calculated with the targeting DP = 50 over conversions.

The first step of our standard purification protocol is the filtration of copolymer solution through basic alumina column to remove Cu species. As compared in Figure S14, the GPC diagram of the copolymer before filtration exhibits the presence of important amount of PEG homopolymers, which could not be covalently attached to PHMssEt blocks. After filtration, the peak equivalent to PEG homopolymer significantly reduced (or disappeared). These results suggest that the ketal linkages labeled in PEG-ketal-Br or PEG-ketal-PHMssEt could be cleaved during ATRP in the presence of Cu(II) species.

Followd by the filtration, the second step is the precipitation of copolymers from hexane (a poor solvent). After precipitates were isolated by a vacuum filtration, the supernatant was analyzed by ¹H NMR and GPC techniques after the removal of solvents. The results shown in Figure S15 indicate that the residue dissolved in hexane is PHMssEt homopolymer. Interestingly, the homopolymer had a high molecular weight with broad molecular weight distribution.

For comparison, a control experiment was conducted with PEG-Br (a similar structure, but with no ketal linkage) for ARGET ATRP of HMssEt under similar conditions with PEG-ketal-Br: $[HMssEt]_0/[PEG-Br]_0/[Cu(II)Br_2]_0/[TPMA]_0/[Sn-(II)EH_2]_0 = 50/1/0.05/0.15/0.4$ in anisole at 40 °C. At the conversion of HMssEt = 0.48, the purified PEG-b-PHMssEt was characterized with the DP of PHMssEt block = 25 by ¹H NMR (Figure S16), which is close to the DP = 24 theoretically estimated with the targeting DP = 50. This result not only confirms the reproducibility of our previous publication,⁷¹ but also confirms the possibility to the cleavage of the ketal linkages labeled in PEG-ketal-Br or PEG-ketal-PHMssEt under ATRP conditions.

Given the above results, a cleavage of ketal linkages was examined for PEG-ketal-Br in the presence of $CuBr_2$ in DMSOd₆ using ¹H NMR spectroscopy. Their concentrations were



Figure 7. Evolution of ¹H NMR spectra and extent of ketal cleavage for a mixtrue of PEG-ketal-Br in the presence of $CuBr_2$ in DMSO- d_6 over time at room temperature.



Figure 8. Schematic illustration of dual-stimuli acidic pH and reduction-responsive cleavage of a ketal linkage and disulfide pendants of DLDSRD (a), ¹H NMR spectrum of DLDSRD-1 treated with a trace amount of HCl (b), and GPC diagrams of DLDSRD-1 in the absence and presence of excess DTT (1.2 equiv to pendant disulfides) and HCl (0.63 mmol) in DMF (c).

designed to be similar to those used for ARGET ATRP above. As seen in Figure 7, the integral of the peak at 1.3 ppm corresponding to two ketal methyl groups decreased, while the integral of the peak at 2.1 ppm to acetone released as a result of the cleavage of ketal linkages increased. From the integrals, the extent of ketal cleavage was quantitatively estimated. It reached >70% within 4 h. Note that no cleavage was found in the absence of CuBr₂. These results suggest that the ketal linkages in PEG-ketal-Br and PEG-ketal-PHMssEt could be cleaved under ATRP conditions.

Aqueous Micellization and Preliminary Investigation of Dual Stimuli Response of DLDSRDs. Given the above

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results, some portion of ketal linkages at block junctions of DLDSRDs were cleaved during ATRP. Nevertheless, both PEG and PHMssEt homopolymers generated as a result of the unexpected cleavage during ATRP could be removed by the established purification process with precipitation and filtration. Consequently, the purified DLDSRD copolymers consist of hydrophilic PEG and hydrophobic PHMssEt blocks.

Because of the amphiphilic nature, the DLDSRD copolymer formed colloidally stable micellar aggregates with the diameter = 198 nm by aqueous micellization through self-assembly in aqueous solution (Figure S17). Further, the purified DLDSRD contains both a ketal linkage at the block junction and disulfide pendants in hydrophobic PHMssEt block. The labile linkages can be cleaved in response to acidic pH and reduction individually or dually (Figure 8a). The feasibility to dual stimuli-responsive degradation of DLDSRD copolymers was examined in organic solution by GPC and ¹H NMR techniques. Note that the kinetics of dual stimuli-responsive degradation of copolymers in organic solution could be different from their nanoassemblies in water. The response of ketal linkage to acidic pH was first examined as an aliquot of DLDSRD-1 was dissolved in CDCl₃ and treated with a trace amount of HCl. The ¹H NMR spectrum in Figure 8b shows the new peak at 2.18 ppm corresponding to acetone released as a consequence of the cleavage of block junction ketal linkages, suggesting the cleavage of ketal linkage in an acidic condition. Similar result was observed for PEG-ketal-Br initiator being treated with HCl (Figure S18). Then, the response to dual acidic pH and reduction was examined as DLDSRD-1 was mixed with an excess DTT (1.2 equiv to pendant disulfide linkages) and HCl (0.63 mmol). As shown in Figure 8c, GPC trace was shifted to lower molecular weight region as a result of the cleavage of both one ketal linkage and 164 disulfide pendants.

CONCLUSION

A new strategy utilizing ATRP was investigated to synthesize PEG-ketal-PHMssEt DLDSRDs consisting of a hydrophilic PEG block covalently conjugated through a ketal linkage with a hydrophobic polymethacrylate block having pendant disulfide linkages. The synthesis of a PEG-ketal-Br macroinitiator was not straightforward because of the unexpected side reactions associated with the high sensitivity of ketal linkage to environments. The carbamate group was turned to be robust to a basic hydrolysis condition compared to ester and carbonate groups. Carbamate chemistry and required protection/deprotection chemistries allow for the development of a robust route with multiple steps to synthesis of PEG-ketal-Br macroinitiator. Under ATRP conditions, the ketal linkages in the macroinitiators and polymeric species were unstable and likely cleaved. This ketal instability could result in the synthesis of PEG-ketal-PHMssEt with 3-4 times higher DP of PHMssEt block compared to theoretically estimated ones (thus, DP of PHMssEt = 100-150). Promisingly, the purified DLDSRDs enabled self-assembly to form nanoassemblies with ketal at core/corona interfaces and multiple disulfide linkages in hydrophobic cores. Further, ¹H NMR and GPC results confirm the dual acidic pH/reductionresponsive cleavage of ketal and disulfide linkages in dual locations.

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.macro-mol.7b02070.

Experimental details; Figures S1-S18 (PDF)

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Notes

The authors declare no competing financial interest.

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