Dual Reduction/Acid-Responsive Disassembly and Thermoresponsive Tunability of Degradable Double Hydrophilic Block Copolymer

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ABSTRACT: We report a thermoresponsive double hydrophilic block copolymer degradable in response to dual reduction and acidic pH at dual locations. The copolymer consists of a poly(ethylene oxide) block covalently connected through an acid-labile acetal linkage with a thermoresponsive polymethacrylate block containing pendant oligo(ethylene oxide) and disulfide groups. The copolymer undergoes temperature-driven self-assembly in water to form nanoassemblies with acetal linkages at the core/corona interface and disulfide linkages in the core, exhibiting dual reduction/acidic responses at dual locations. The physically assembled nanoaggregates are converted to disulfide-core-crosslinked nanogels through disulfide–thiol exchange reaction, retaining enhanced colloidal stability, yet degraded to water-soluble unimers upon reduction/acidic-responsive degradation. Further, the copolymer exhibits improved tunability of thermoresponsive property upon the cleavage of junction acetal and pendant disulfide linkages individually and in combined manner. This work suggests that dual location dual reduction/acid-responsive degradation is a versatile strategy toward effective drug delivery exhibiting disulfide-core-crosslinking capability and disassembly as well as improved thermoresponsive tunability.

INTRODUCTION

Amphiphilic block copolymers (ABPs) have been extensively explored as building blocks in the development of polymer-based drug delivery systems.1–6 Well-defined ABPs consist of a hydrophobic block covalently conjugated with a hydrophilic block, typically poly(ethylene glycol) (PEG). Because of their amphiphilicity, they undergo self-assembly in aqueous solution to form nanoassemblies with hydrophobic cores, capable of encapsulating bioactive molecules. The integration of cleavable covalent linkages to facilitate stimuli-responsive degradation through chemical transition enhances the release of encapsulated bioactive molecules.5–11 Among chemical and physical stimuli, reduction12–15 and acidic pH18,19 are found in biological systems for attaining biodegradation. Typically, tumor microenvironments have an elevated concentration of cellular glutathione (a tripeptide)16 and are slightly acidic (pH = 6.5–6.9).17 Even more acidic environments (pH = 4.5–5.5) can be found in endosomes and lysosomes. Reduction-responsive degradable nanoassemblies have been developed by incorporating disulfide bonds as a pendant chain in the hydrophobic block of ABPs, facilitating the formation of core-degradable nanoassemblies.18–22 This approach is promising because the physically associated nanoassemblies can be converted to the corresponding disulfide-core-crosslinked nanogels through disulfide–thiol exchange reaction, providing improved colloidal stability.23–25 These disulfide pendants and cross-links can be reduced to their corresponding thiol (–SH) forms only in the presence of cellular glutathione. This reductive degradation process can result in the change of hydrophobic/hydrophilic balance, causing disintegration of the nanoassemblies, leading to enhanced release of encapsulated bioactive molecules. However, while the degraded products are labeled with a hydrophilic functional group, it is not sufficient to render the pendant chain water-soluble; thus, they tend to form large aggregates. This presents a critical challenge in the removal from the body after action, which may stem from the hydrophobic nature of ABPs.

Double hydrophilic block copolymer (DHBC) is a class of the block copolymers composed of both water-soluble and hydrophilic blocks.26,27 Particularly, a thermoresponsive DHBC designed with a thermoresponsive block exhibits a switchable amphiphilic nature upon a change in temperature. Poly(N-isopropylacrylamide) and poly(oligo(ethylene oxide) monomethyl ether methacrylate) are commonly used thermoresponsive polymers. These thermoresponsive blocks can undergo a transition from a hydrophilic to hydrophobic...
state above a specified temperature, whereas another hydrophilic block composed of mainly PEG remains water-soluble. Through the unique temperature response, thermoresponsive DHBCs self-assemble to form nanoaggregates at temperatures above its lower critical solution temperature (LCST). Their LCST can be adjusted with the change in molecular weight and degree of hydrophobicity of thermoresponsive blocks. These features have made thermoresponsive DHBCs useful as effective building blocks for in situ gelation, catalysis, cellular imaging, and delivery. However, the physically assembled nanoaggregates are dissociated to unimers when the temperature decreases below LCST. Such instability in aqueous solution against heating remains challenging.

An effective strategy to achieve enhanced colloidal stability is the integration of cross-linking chemistry in the design of thermoresponsive DHBCs, leading to the synthesis of cross-linked nanogels. The use of labile cross-links such as disulfide linkages offers further reduction-responsive disassembly of the formed bioreducible nanogels. Reports describe the fabrication of bioreducible nanogels cross-linked with disulfide cross-links through the incorporation of disulfide-bearing cross-linkers. As an example, a thermoresponsive PEG-based block copolymer consisting of poly(NIPAM-co-N-acryloxy succinimide) was synthesized. Cystamine was used as an external cross-linker bearing a disulfide to achieve disulfide core-crosslinking through facile coupling reaction. Further, few reports describe the synthesis of bioreducible nanogels through disulfide−thiol exchange reaction of a thermoresponsive polymethacrylate bearing pendant disulfide linkages. However, most of the reported bioreducible systems exhibit single stimulus-responsive disassembly in response to reduction. In this context, the exploration of novel strategies to incorporate acid-labile linkage to core/corona interfaces is highly desired to develop novel bioreducible nanogels exhibiting dual reduction/acid responses in dual locations (cores and interfaces) for precise control over assembly/disassembly as well as thermoresponsiveness.

In this work, we have developed a strategy utilizing atom transfer radical polymerization (ATRP) to synthesize a thermoresponsive DHBC degradable in response to dual reduction/acid stimuli. The block copolymer consists of the PEG block connected through an acid-labile acetal linkage with a thermoresponsive methacrylate-based random copolymer block containing pendant oligo(ethylene oxide) and disulfide moieties, thus forming PEG-acetal-P(OEOMA-co-HMssEt) (PKM) (HMssEt: a methacrylate having a pendant disulfide linkage). The formed PKM copolymer exhibits the tunable thermoresponsive properties with varying densities of hydrophobic HMssEt units in the thermoresponsive block. Temperature-driven self-assembly and following interchain disulfide−thiol exchange reaction allow for the fabrication of disulfide-core-crosslinked bioreducible nanogels with acetal linkages at core/corona interfaces, attaining dual reduction/acid responses at dual locations. The formed nanogels were characterized for dual acid/reduction-responsive disassembly as well as in vitro cell viability and cellular uptake toward potential for drug delivery. Further, the versatility of dual reduction/acid-responsive degradation was studied for the tunability of LCST for the copolymer.

### RESULTS AND DISCUSSION

#### Synthesis of Copolymers and Kinetics Investigation.

Our experiments began with the synthesis of an acetal-labeled macroinitiator of PEG-ac-Br. As depicted in Figure 1a, the first step was the synthesis of a vinyl ether bromide (VE-Br) by a facile esterification of ethylene glycol vinyl ether with α-bromoisobutyryl bromide at 90% yield as described elsewhere. Its chemical structure was confirmed by 1H NMR analysis (Figure 1b). The next step was the acetal formation through the reaction of the formed VE-Br (30 mol equivalent excess) with PEG in the presence of pyridinium p-toluenesulfonate (PPTS) catalyst, followed by purification from diethyl ether. The 1H NMR spectrum in Figure 1b shows the presence of PEG moieties at 4.0−3.2 ppm (g and EOs), bromoisobutyryl group at 1.9 ppm (a), and acetal moieties at 1.4 ppm (d), confirming the synthesis of well-defined PEG-ac-Br at 84% yield.

After successful synthesis and characterization, the formed PEG-ac-Br was used as a macroinitiator for ATRP of a mixture of HMssEt (a methacrylate having a pendant disulfide) with oligo(ethylene oxide) monomethyl ether methacrylate (OEMA). As illustrated in Figure 2a, the activators regenerated by Electron Transfer (ARGET) process for ATRP was examined with CuBr2/tris(2-pyridylmethyl)amine (TPMA) complex and tin(II) 2-ethylhexanoate (Sn(II)(EH)2) reducing agent in anisole at 40 °C. With an initial mole ratio of [OEMA + HMssEt]0/[PEG-ac-Br]0 = 70/1, projecting the degree of polymerization (DP) to be 70 at complete monomer conversion, the amounts of HMssEt in the feed were varied to synthesize a series of block copolymers with different densities of disulfide pendants between 0 and 30 mol %.

Polymerization was stopped to reach monomer conversions within the range of 55−70%. The formed copolymers were purified by precipitation from hexane to remove residual monomers and passed through basic aluminum oxide to remove residual metal species. Then, the purified, dried copolymers were characterized to confirm their chemical structures and measure their molecular weight. As an example, for PKM-30 containing 30 mol % disulfide pendants, 1H NMR
in Figure 2b shows the characteristic peaks corresponding to methyl groups on backbones between 0.8 and 1.2 ppm (a, a’) and pendant methoxy groups of OEOMA units at 3.3–3.8 ppm (EO) as well as methylene groups adjacent to disulfide groups in HMssEt units at 2.9 ppm (h, i). Using their integrals, the mol % of HMssEt units in the formed PKM-30 was estimated to be 33. In a similar way, the HMssEt mol % for other copolymers was estimated. Gel permeation chromatography (GPC) was used to determine molecular weight of the purified, dried copolymers (Figure S1). They had the number average molecular weight ($M_n$) = 23–27 kg/mol with narrow dispersity as $D < 1.3$. These results are summarized in Table 1.

To investigate the kinetics of ARGET ATRP for a mixture of HMssEt and OEOMA in the presence of PEG-ac-Br, aliquots were taken during PKM-30 polymerization to determine monomer conversion and molecular weights. As seen in Figure S2, the polymerization was first-order with constant concentration of active radicals, molecular weight increased linearly with conversion, and dispersity remained low ($D < 1.2$). Further, molecular weight distribution evolved to high molecular region over conversion. These results suggest that ARGET ATRP of HMssEt and OEOMA in the presence of PEG-ac-Br is well-controlled.

Investigation of Thermoresponsive and Solution Properties. The thermoresponsive properties of the formed double-hydrophilic block copolymers were examined using dynamic light scattering (DLS) for aqueous solutions of PKM copolymers dissolved in phosphate buffer saline (PBS) solution at pH = 7.4 at 0.1 wt %. As seen in Figure 3, the normalized light scattering intensity remained low at lower temperatures, which is attributed to the existence of copolymers as unimers in aqueous solution. Upon an increase in temperature, the intensity abruptly increased, which is attributed to the aggregation of polymeric chains through the change in their polarity. Such change in hydrophilic/hydrophobic balance confirms that the formed double hydrophilic copolymers are thermoresponsive. The LCST is defined as the temperature at which the normalized light scattering intensity starts to drastically decrease with an increasing amount of hydrophobic HMssEt units in the polymethacrylate block. For example, the LCST was 76.1 °C for PKM-0 with no HMssEt units but decreased to 61.8 °C with 10 mol % hydrophobic HMssEt units for PKM-10 and further to 40.3 °C with 30 mol % HMssEt units for PKM-30.

Given that PKM-30 has its LCST at 40 °C, close to the body temperature, the copolymer was chosen to demonstrate a temperature-driven assembly and disassembly via DLS. An aliquot of PKM-30 dissolved in PBS (pH = 7.4) at 0.3% wt was subjected to a cyclic change between 10 and 60 °C, a temperature below and above its LCST, respectively. The DLS diagrams of size distributions by volume in Figure 4 and by

Table 1. Characteristics and Properties of PKM Copolymers Prepared by ARGET ATRP in the Presence of PEG-ac-Br

| copolymers | HMssEt (mol %) | Conv $^b$ | $M_n$ (kg/mol) | $M_w/M_n$ $^c$ |
|------------|----------------|-----------|----------------|----------------|
| PKM-0      | 0              | 0.55      | 23.3           | 1.13           |
| PKM-10     | 10             | 0.62      | 25.5           | 1.15           |
| PKM-30     | 30             | 0.68      | 27.2           | 1.12           |

$^a$Conditions for ATRP: [OEOMA + HMssEt]$_o$/[Cu(II)Br$_2$]$_o$/[TPMA]$_o$/[Sn(II)EH$_2$]$_o$ = 70/0.05/0.15/0.4 in anisole at 40 °C.
$^b$By 1H NMR. $^c$By GPC with polymethyl methacrylate standards.

Figure 2. Scheme illustration utilizing ARGET ATRP to synthesize PKM copolymers (a) and $^1$H NMR spectrum in CDCl$_3$ of PKM-30 as an example (b).

Figure 3. Normalized count rate by DLS for PKM-0, PKM-10, and PKM-30 in aqueous solution over temperature.

Figure 4. DLS diagrams by volume at 10 and 60 °C to demonstrate reversible self-assembly/disassembly of PKM-30.
intensity in Figure S3 are shown. At 10 °C below its LCST, the intensity distribution shows the coexistence of two components; however, the volume distribution indicates the existence of most copolymer chains as unimers with the average hydrodynamic diameter to be 9 nm. When temperature increased to 60 °C above its LCST, both intensity and volume distribution show one population with the diameter by volume to 46 nm. Such an increase in the diameter could be attributed to temperature-driven aggregation. Because of physical association of the copolymer chains, the formed aggregates turned to unimers when the temperature decreased to 10 °C. Such a reversible temperature response is further demonstrated through repeated heating—cooling cycles at 60 and 10 °C.

Investigation of Acid/Reduction-Responsive Degradation. The copolymers are labeled with pendant disulfide linkages in the polymethacrylate block and an acetal linkage at the block junction. The cleavage of these labile linkages in response to acid and reduction stimuli was investigated with PKM-30 fully dissolved in dimethylformamide (DMF). Figure 5a shows the schematic illustration of single acid or reduction response and dual responses of PKM copolymer to acidic pH/reduction stimuli. GPC was mainly used to follow the degradation (Figure 5b). When being incubated with acid (HCl), PKM-30 degraded to PEG-OH, acetaldehyde, and PKM-OH upon the cleavage of the acetal linkages at block junctions. GPC analysis confirms the decrease in molecular weight to \( M_n = 17.7 \) kg/mol from \( M_n = 27.2 \) kg/mol, and the shift of GPC trace to lower molecular region between PKM-30 and PEG-ac-Br (macroinitiator). A shoulder in lower molecular weight region corresponds to that of PEG-ac-Br macroinitiator, confirming the generation of PEG-OH as a degraded product. Upon exposure to the reducing agent dl-dithiothreitol (DTT) (5 mol equivalent), the disulfide pendants in the polymethacrylate block could be cleaved, generating PKM-SH as a possible degraded macromolecular product. Its molecular weight slightly decreased from 27.2 kg mol\(^{-1}\) \( (D = 1.1)\) to 20.3 kg mol\(^{-1}\) \( (D = 1.3)\), confirming reductive cleavage of significant densities of pendant disulfide linkages. In the presence of DTT at acidic pH (dual stimuli), GPC analysis shows the decrease in molecular weight to 16.1 kg/mol and the shift of molecular weight distribution to low molecular region between PKM-30 and PEG-ac-Br. Such a decrease in the molecular weight could be attributed to the generation of PEG-OH and PKM-OH-SH as degraded macromolecular products upon the cleavage of junction acetal and pendant disulfide linkages.

In Situ-Disulfide-Core-Crosslinking and Dual Acid/Reduction-Degradable Disassembly. The nanoaggregates formed at the temperature above LCST by temperature-driven self-assembly in aqueous solutions consist of P(OEOMA-co-HMssEt) cores surrounded with PEG coronas. They, however, could be dissociated to the corresponding unimers upon a decrease in temperatures below LCST. To enhance colloidal stability at room temperature, the pendant disulfide linkages in PKM-based cores could be utilized to cross-link the nanoaggregates through disulfide–thiol exchange reactions in the presence of a catalytic amount of reducing agent, yielding the corresponding nanogels cross-linked with new disulfide linkages. This process, called in situ-disulfide core-crosslinking, was examined by incubation of nanoaggregates with a catalytic amount of DTT (0.2 mol equivalent) at 60 °C for 12 h. After being cooled to room temperature, the formed products were analyzed by DLS and transmission electron microscopy (TEM) techniques. The DLS diagram shows the diameter by volume = 50 nm (Figure 5a), which is somewhat larger than that (46 nm) of uncrosslinked counterparts measured at 60 °C. Note that the diameter was 9 nm for uncrosslinked nanoaggregates measured at 10 °C (see Figure 4). TEM analysis confirms the diameter = 16.3 ± 4.3 nm, which is smaller than that determined by DLS (Figure 5a inset). This is attributed to the dehydrated state of nanogels on TEM grids as well as the consequence of cross-linking. Further, the diameter
remained unchanged upon 1000-fold dilution in PBS (Figure 6b). These combined results suggest the occurrence of in situ disulfide-core-crosslinking with enhanced colloidal stability.

As illustrated in Figure 6d, the formed disulfide-core-crosslinked nanogels are labeled with an acetal linkages at the interfaces and disulfide pendants and cross-links in the cross-linked cores. These linkages could be cleaved in response to both acid and reduction, resulting in the degradation of the nanogels in aqueous solution. To investigate dual acid/reduction-responsive degradation, the nanogels were incubated with 10 mM DTT at acidic pH = 4.5. As seen in Figure 6c, the diameter significantly decreased to 5 nm after 1 h. Such rapid degradation could be a consequence of both reductive cleavage of disulfides and acid-cleavage of acetal linkages. The detected species, likely the fully degraded product PKM-OH-SH, appears to be fully water-soluble and together with its smaller size. This result could be promising to facilitate the excretion of the degraded products from body.

To further investigate the degradation to single response, the nanogels were incubated in acidic pH and DTT separately. Summarized in Figure S4, the diameter increased to >250 nm in an acidic environment, which could be attributed to the possible aggregation of disulfide-crosslinked cores upon the detachment of PEG coronas. When the nanogels were incubated with 10 mM DTT, their diameter significantly decreased to 7 nm after 24 h as a consequence of the reductive degradation to yield PKM-SH, which could be also dissolved in water.

**Biological Evaluation with Cell Viability and Intracellular Trafficking.** For a preliminary evaluation of disulfide-core-crosslinked nanogels for biological applications, first their cytotoxicity was examined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with HeLa cancer cells. As seen in Figure 7, the nanogels had the viability to be >85%, suggesting that they are not toxic to the cells up to the concentration of 400 μg/mL. Further, the nanogels were examined for their intracellular tracking by epi-fluorescence microscopy (Figure 8). Doxorubicin (Dox)-loaded disulfide-core-crosslinked nanogels were fabricated and purified by the intensive dialysis method to remove free Dox molecules. The formed nanogels had the diameter = 168 nm on average by DLS analysis and 3.3% of loading level by UV/vis spectroscopy (Figure S5). HeLa cells incubated with Dox and Dox-loaded nanogels show a strong fluorescence signal inside the cells and around the perinuclear region. This indicates that both Dox and Dox-loaded nanogels were taken up by the cells. Further, a stronger Dox fluorescence signal from cells treated with Dox-loaded nanogels indicate that the micelles were taken up by the cells to a greater extent in comparison to free Dox.

**Acid/Reduction-Responsive Tunability of Thermoresponsive Properties.** Dual acid/reduction-responsive degradation was further investigated for the tunability of thermoresponsive properties of the copolymer. PKM-10 containing 10 mol % hydrophobic HMsEt was examined for DLS analysis (Figure 9). GPC analysis confirms the significant cleavage of acetal and disulfide linkages under acidic, reductive, and both conditions (Figure S6) to form the possible degraded products (see Figure 5a). When the PKM-10 was incubated in acidic pH, the LCST of PKM decreased from 61.8 to 51.9 °C (PKM-OH) by 10 °C. Such a decrease can be attributed to the
loss of water-soluble PEG block upon the cleavage of junction acetal linkage. In the reductive environment, the LCST of PKM increased to 70 °C (PKM-SH), which is presumably due to the increase in polarity upon the cleavage of disulfide pendant to more hydrophilic thiol groups. When the copolymer was treated with both acidic pH and reduction, the LCST of PKM-OH-SH was determined to be 57.5 °C. It is lower by 13 °C than PKM-SH (single reduction) but greater by 6 °C than PKM-OH (single acid). These results suggest that thermoresponsive properties of the double hydrophilic copolymers can be tuned with the change in both hydrophilicity and hydrophilic/hydrophobic balance upon the stimuli-responsive cleavage of labile linkages at the block junction and in hydrophobic pendant chains.

**CONCLUSIONS**

A new approach utilizing ATRP enabled the synthesis of a thermoresponsive DHBC consisting of PEG block connected through an acetal linkage with a thermoresponsive poly(methacrylate) block, yielding PEG-acetal-P(OEOMA-co-HMssEt), capable of degrading in response to dual reducing and acidic environments. Its thermoresponsive property as LCST decreased with an increasing density of hydrophobic HMssEt unit up to 30 mol %. These block copolymers formed nanoaggregates through temperature-driven self-assembly in aqueous solution and further converted to disulfide-core-crosslinked nanogels through disulfide–thiol exchange reaction providing enhanced colloidal stability. Dual reductive response in cores and acid response at core/corona interfaces promoted the disassembly of the formed nanogels to water-soluble species, anticipating enhanced release of bioactive molecules and facile removal of degraded products from action sites. The results from cell culture experiments suggest low cytotoxicity and good cellular uptake. Further, the copolymer exhibits improved tunability of its thermoresponsive property upon reduction and acid degradation individually or simultaneously.

**EXPERIMENTAL SECTION**

**Instrumentation.** 

$^1$H NMR spectra were recorded using a 500 MHz Varian spectrometer. The CDCl$_3$ singlet at 7.26 ppm was 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 by $^1$H NMR analysis and molecular weight and its distribution by GPC were determined. An Agilent GPC equipped with a 1260 Infinity Isocratic Pump and an RI detector as well as two Agilent PLgel mixed-C and mixed-D columns run with DMF containing 0.1 mol % LiBr at 50 °C at a flow rate of 1.0 mL/min. Molecular weights were calibrated with linear poly(methyl methacrylate) standards from Fluka. Clear solution of aliquots of the polymers dissolved in DMF/LiBr was filtered using a 0.40 μm polytetrafluoroethylene filter to remove any DMF-insoluble species. A drop of anisole was added as a flow rate marker. The size of nanoaggregates in hydrodynamic diameter ($D_h$) was measured by 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. TEM images were obtained using a Philips Tecnai 12 TEM, operated at 80kV and equipped with a thermionic LaB$_6$ filament. An AMT V601 DVC camera with point-to-point resolution and line resolution of 0.34 and 0.20 nm, respectively, was used to capture images at 2048 by 2048 pixels. To prepare specimens, aqueous dispersions were added

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Figure 8. Fluorescence microscopy images of HeLa cells incubated with (a) free Dox (20 μg/mL) and (b) Dox-loaded nanogels (encapsulated Dox = 20 μg/mL) for 24 h.

Figure 9. Normalized light scattering intensity by DLS in aqueous solution over temperature for PKM-10 and its degraded products formed after the incubation with acid, DTT, and both.
dropwise onto copper TEM grids (400 mesh, carbon coated), blotted, and allowed to air dry at room temperature.

**Measurements of Thermoresponsive Properties of PKM Using DLS.** The purified, dried polymer (10 mg) dissolved in PBS (pH = 7.4, 10 mL) was stabilized overnight to form aqueous clear solutions at 1.0 mg/mL (0.1 wt %). After being filtered through the 0.45 μm polyethersulfone filter to remove unexpectedly formed large aggregates, aliquots were transferred to a quartz cuvette and then sealed with a Teflon stopper. Their changes in light scattering intensity were measured in triplicates at an increment of 1 °C at a temperature range of 10–90 °C.

**Materials.** Triethylamine (Et3N, 99.5%), PPTS (98%), copper(II) bromide (CuBr2, >99.99%), tin(II) 2-ethylhexanoate (Sn(II)(EH)2, 95%), DTT (>98%), and doxorubicin hydrochloride (Dox, (CH3)C(CH3)), 3.77 ppm (m, 2H, −CH2OC(O)−), 3.64 ppm (s, 4H, −OCH(CH3)O−), 3.38 ppm (s, 3H, CH3O−), 3.27 ppm (m, 2H, −CH2OCH2−), 3.13 ppm (d, 3H, −OCH(CH3)O−). The purifications were then performed. PKM-30 (650 mg) dissolved in DMF (9 mL) was mixed with DTT (64 mg, 0.4 mmol) for dual degradation, sequential acid-degradation followed by reduction was performed. PKM-30 (650 mg) dissolved in DMF (9 mL) was mixed with DTT (385 mg, 2.4 mmol) for reductive degradation and both acid and reductive degradation under stirring. DLS analysis was conducted to follow the degradation of nanogels.

**Synthesis of PEG-ac-Br.** An organic solution consisting of PEG (4.3 g, 0.85 mmol) distilled from toluene (15 mL) and PPTS (20 mg, 0.1 mmol) dissolved in dichloromethane (40 mL) was mixed with a solution of VE-Br (6.1 g, 26.5 mmol) in dichloromethane (10 mL) under stirring at room temperature for 72 h. After the addition of Et3N (250 μL, 3.4 mmol) to quench the reaction, the resulting mixture was washed with aqueous PBS solution (pH = 7.4, 50 mL) four times and then precipitated from anhydrous diethyl alcohol to remove inhibitors. TPMA and a methacrylate having a pendant disulfide linkage (HMssEt) were synthesized as described elsewhere.

**General Procedure for ATRP To Synthesize Block Copolymers.** PEG-ac-Br, OEOA, HMssEt, TPMA, CuBr2, and anisole were mixed in a 25 mL Schlenk flask and deoxygenated by nitrogen purging for 1 h in an oil-bath heated to 40 °C. Following purging in nitrogen, a prepurged solution of Sn(II)(EH)2 in anisole was injected into the Schlenk flask to initiate polymerization. Polymerization was stopped by cooling down the solution to room temperature in an ice-bath and exposing it to air. For kinetic studies, aliquots were withdrawn periodically to analyze conversion and molecular weight.

**Synthesis.** PEG-ac-Br (0.5 g, 0.1 mmol), OEOA (2.0 g, 6.7 mmol), TPMA (4.2 mg, 14.3 μmol), [Cu(II)TPMA-Br]L (2.5 mg, 4.8 μmol), anisole (6.7 g), and Sn(II)(EH)2 (15.5 mg, 38.2 μmol) for PKM-0; PEG-ac-Br (0.5 g, 0.1 mmol), OEOA (1.8 g, 6.0 mmol), HMssEt (0.2 g, 0.7 mmol), TPMA (4.2 mg, 14.3 μmol), [Cu(II)TPMA-Br]L (2.5 mg, 4.8 μmol), anisole (6.6 g), and Sn(II)(EH)2 (15.5 mg, 38.2 μmol) for PKM-10; and PEG-ac-Br (0.5 g, 0.1 mmol), OEOA (1.4 g, 4.7 mmol), HMssEt (0.7 g, 1.83 mmol), TPMA (4.2 mg, 14.3 μmol), [Cu(II)TPMA-Br]L (2.5 mg, 4.8 μmol), anisole (7.1 g), and Sn(II)(EH)2 (15.5 mg, 38.2 μmol) for PKM-30. For purification, the as-synthesized solutions were diluted with acetone (150 mL), passed through a basic aluminum oxide column to remove residual Cu species and ligand, and precipitated from hexane to remove unreacted monomers. The resultant precipitates were dried in a vacuum oven for overnight.

**Reversible Assembly/Disassembly upon Temperature Change.** The purified, dried PKM-30 (10 mg) was dissolved in PBS (pH = 7.4, 10 mL) at room temperature. An aliquot of the resulting aqueous solution (1.5 mL) was transferred to a quartz cuvette and kept in a water-bath pre-set at 10 °C for 30 min and then 60 °C for 30 min. DLS analysis was conducted to determine the size in diameter by volume upon change in the temperature.

**Investigation of Acid/Reduction-Responsive Degradation in DMF.** For acid degradation, PKM-30 (650 mg) dissolved in DMF (9 mL) was mixed with 15 N HCl (50 μL, [H+] = 82 mM). For reductive degradation, PKM-30 (100 mg) dissolved in DMF (8 mL) was mixed with DTT (64 mg, 0.4 mmol). For dual degradation, sequential acid-degradation followed by reduction was performed. PKM-30 (650 mg) dissolved in DMF (9 mL) was mixed first with 15 N HCl (50 μL, [H+] = 82 mM) and then following DTT (385 mg, 2.4 mmol). GPC was used to follow any changes in molecular weight and its distribution. Note that the concentrations of junction acetal and pendant disulfide linkages in PKM-30 were estimated with monomer conversion for DP and 2-hydroxyethyl methacrylate/OEOA mole ratio by NMR analysis.

**In Situ-Disulfide-Core-Crosslinking to Nanogels.** PKM-30 (21 mg, 0.02 mmol of pendant disulfide linkages) was dispersed in PBS (pH = 7.4, 6 mL) for a concentration of 0.3 wt % at 60 °C for 2 h and then mixed with an aqueous solution of DTT (0.5 mg, 0.2 mol equivalent to disulfides) in PBS (pH = 7.4, 1 mL) for 24 h. For purification, the resulting dispersion of disulfide-core-crosslinked nanogels was dialyzed against PBS buffer (pH = 7.4) for 24 h. DLS analysis was conducted at 10 °C.

**Dual Acid/Reduction-Degradable Disassembly of Disulfide-Core-Crosslinked Nanogels.** An aqueous disulfide-core-crosslinked PKM-30 nanogel dispersion (3 mL, 2 mg/mL) was mixed with aqueous acetate buffer solution (15 mL, pH = 4.5) for acid degradation, DTT (15.7 mg, 0.1 mmol) for reductive degradation and both for dual degradation under stirring. DLS analysis was used to follow the degradation of nanogels.

**Encapsulation of Dox.** An aqueous solution of PKM-30 (21 mg) dissolved in PBS (pH = 7.4, 5 mL) was mixed with an organic solution of Dox (2.0 mg) and Et3N (2.0 mg) dissolved in DMF (1.0 mL). The resulting mixture was heated to 60 °C at which was treated with DTT (0.6 mg, 0.2 mol equivalent to disulfides) for 24 h. After being cooled to room temperature, the resulting dispersion was purified by dialysis against PBS buffer (pH = 7.4, 1 mL) for 13 h. Outer PBS was changed over 7 times to remove residual (not encapsulated) Dox molecules in the dispersion. In this way, Dox-loaded disulfide-core-crosslinked nanogels dispersions were prepared at 3.5 mg/mL. Loading level of Dox was determined by UV/vis spectroscopy with the extinction coefficient of Dox (ε = 12,400 M⁻¹·cm⁻¹) determined in a mixture of water/DMF (1/5 v/v) at λmax = 497 nm.

**Cytotoxicity by MTT Assay.** HeLa cells cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10 vol % fetal bovine serum and 1 vol % penicillin-streptomycin.
solution was harvested at 80% confluency, and $6 \times 10^5$ cells were seeded into each well of 96 well plate. After incubation at 37 °C for 24 h, the media was replaced with DMEM (100 μL) containing different concentrations of micellar dispersion, and the cells were further incubated at 37 °C for 48 h. Untreated cells as NC and cells treated with 1% Triton-X100 as PC were used. The media was then replaced with DMEM (100 μL) containing 10% MTT dye and incubated at 37 °C. After 4 h, MTT solution was carefully removed and the formazan crystals were dissolved in dimethylsulfoxide (200 μL). The absorbance at 570 nm was measured using Tecan Infinite M200 PRO microplate reader, and the cell viability was calculated using the formula. Cell viability was calculated as the percent ratio of absorbance of mixtures with micelles to control (cells only without NPs).

**Cellular Uptake by Epifluorescence Microscopy.** HeLa cells were seeded at a concentration of $1 \times 10^5$ cells/mL into a 35 mm glass bottom plate and cultured in DMEM. After 24 h, the media was replaced with DMEM containing either Dox or Dox-loaded micelles such that the final concentration of Dox in the micelles was 20 μg/mL. After a 4-h incubation, the cells were washed with PBS (3 times) and PBS containing 2 μL of Hoechst 33342 (5 mg/mL) added to the cells to stain the nucleus. After 15 min incubation in the dark, the stain solution was replaced with phenol-red free DMEM and the cells were observed by Nikon Eclipse TiE inverted epifluorescence microscope.

**Investigation of Thermoresponsive Properties upon Acid/Reduction-Responsive Degradation.** PKM-10 co-polymer was incubated with HCl, DTT, and both as described previously. The formed degraded products were purified by precipitation from cold diethyl ether. The resultant precipitates were dried in a vacuum oven without precipitation from cold diethyl ether. The resultant precipitates were observed by Nikon Eclipse TiE inverted epifluorescence microscope.

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Notes

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