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Thiol-responsive hydrogel scaffolds for rapid change in thermoresponsiveness†

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A facile strategy to fabricate thiol-responsive thermoresponsive hydrogels able to rapidly change their volume in response to temperature is reported. The strategy utilizes crosslinking atom transfer radical polymerization to synthesize well-defined hydrogels of thermoresponsive oligo(ethylene oxide)-based polymethacrylates with a uniform network crosslinked with dynamic disulfides. Thiol-responsive cleavage of disulfide linkages to the corresponding pendant thiols allows for the generation of hydrophilic dangling chains in the hydrogels as well as the increase in hydrophilicity of the hydrogel network. The degraded hydrogels exhibit a rapid change of thermoresponsiveness (deswelling kinetics) with a slight sacrifice in mechanical properties. Evaluating the hydrogels from a biomedical perspective, rapid thermoresponsive hydrogels are non-cytotoxic and exhibit enhanced release of encapsulated model drugs.

Introduction

Hydrogels are three-dimensional crosslinked networks of hydrophilic polymers with tunable chemical and physical structure, good mechanical properties, high water content, and biocompatibility.[1–4] Stimuli-responsive hydrogels can change their dimensions in response to various external stimuli.[5–9] In particular, thermoresponsive hydrogels can swell or deswell in response to changes in temperature, thus undergoing a volume change at low critical solution temperature (LCST) in water. Above the LCST, the hydrogels are hydrophobic and expel water; below the LCST, they are hydrophilic and swollen in water.[10–12] These thermoresponsive properties, combined with the features of normal hydrogels, offer great potential for the utilization of thermoresponsive hydrogels in tissue engineering[13,14] and drug delivery,[15,16] as well as recently biosensors,[17–19] intracellular thermometry,[20] photonic crystals,[21] and bioconjugates.[22,23]

One desirable characteristic of thermoresponsive hydrogels is the ability to rapidly change their volume in response to temperature. A number of studies indicate that the limiting factor in thermoresponsive hydrogels is the formation of an impenetrable hydrophobic "skin layer" on the surface of the material, and this layer interferes with the release of water from the hydrogel. This delayed water release has been attributed to the slow LCST transition of thermoresponsive polymers from hydrophilicity to hydrophobicity. Rapid response is achieved by fast water release from the gel matrix, while preventing formation of skin layers on hydrogel surface caused by LCST transition of thermoresponsive polymers. Several approaches have been reported to accelerate hydrogel response rate. Most approaches are designed based on the introduction of hydrophilic chains or domains to enhance the release rate of water from hydrophobic thermoresponsive hydrogels. These approaches include grafting dangling chains with surface-active molecules,[24–27] adjusting hydrophilic/hydrophobic balance,[28] and forming interpenetrating polymer networks (IPN)[29] and heterogeneous crosslinked networks.[30–33] Another approach involves the formation of porous structures within the hydrogels. These micropores can be formed by sacrificing embedded molecules such as sucrose,[34] inorganic nanoparticles,[35] and recently degradable star polymeric nanoparticles.[36] None of these approaches appear to offer broad applicability; however, they are often associated with an undesirable decrease of the overall hydrogel swelling ratio.

Stimuli-responsive degradation (SRD) is a dynamic and powerful platform that involves the cleavage of covalent bonds in response to external stimuli such as low pH, light, or ultrasound, as well as reductive, oxidative, or enzymatic reactions.[37,38] Disulfides are cleaved to the corresponding thiols in a reducing environment or through a disulfide–thiol exchange.[39] Disulfide–thiol degradation chemistry offers an advantageous SRD platform in constructing reductively-responsive degradable nanomaterials desirable for various biomedical applications; these include self-assembled micellar nanocarriers,[40,41] nanocapsules,[42] nanogels,[43–45] hydrogels,[36,46] and bioconjugates.[47–49] Furthermore, this type of degradation chemistry has been explored for tuning lower critical solution temperature (LCST) of polymeric materials[50,51] and changing morphologies of self-assembled nanostructures.[52] Moreover, disulfide crosslinked-hydrogels that can undergo reductively-responsive cleavage would be interesting as smart precursors for the synthesis of unique thermoresponsive nanomaterials with rapid change of thermoresponsive properties.

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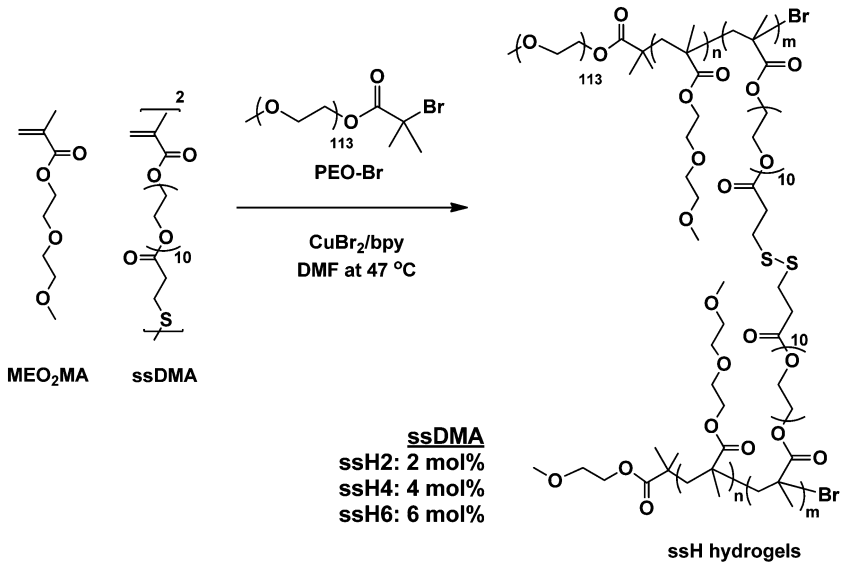
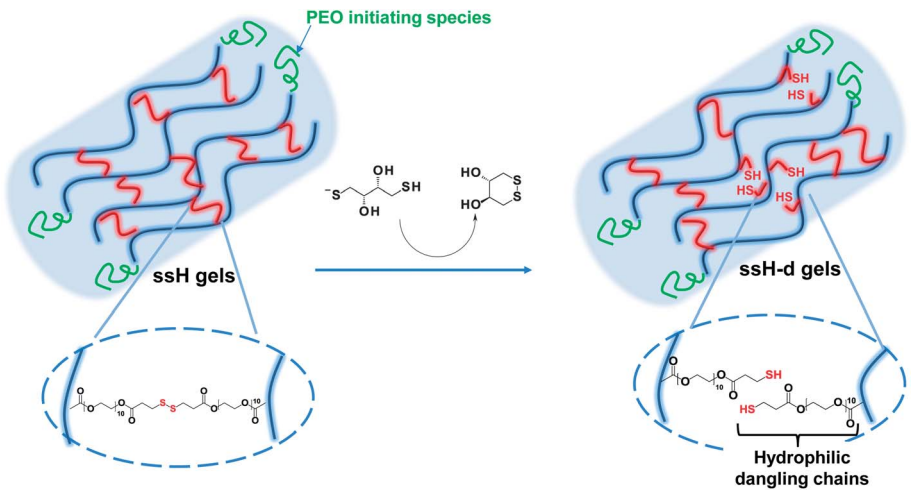


Scheme 1 Synthesis of $PMEO_2MA$-based thermoresponsive hydrogels crosslinked with disulfide linkages by AGET ATRP of MEO_2MA in the presence of various amounts of ssDMA in DMF at 47 °C.



Scheme 2 A facile strategy utilizing disulfide–thiol degradation chemistry to synthesize thiol-responsive hydrogel scaffolds for enhanced thermoresponsive properties. ssH gels and ssH-d gels denote as cleavable hydrogels and degraded ssH gels, respectively.

Here we describe a facile strategy utilizing disulfide–thiol degradation chemistry to synthesize effective hydrogels exhibiting rapid change in thermoresponsive properties. Thermoresponsive polymethacrylate containing pendant oligo(ethylene glycol) (POEOMA) was targeted as the scaffold materials.[53,54] POEOMA is an analog of poly(ethylene oxide) (PEO); PEO is biocompatible material that has been FDA-approved for clinical use, has low toxicity, and prevents nonspecific protein adsorption.[55,56] The precursor thermoresponsive hydrogels labeled with disulfide linkages were synthesized by controlled radical polymerization (CRP)[57] in the presence of a disulfide-labeled dimethacrylate (ssDMA) (Scheme 1). The CRP ensures the formation of uniform network with higher swelling ratios, compared to their counterparts prepared by conventional free radical polymerization (FRP).[58,59] As illustrated in Scheme 2, the reductive cleavage of disulfides in the precursor hydrogels to the corresponding thiols (SH groups) generates short dangling chains in the hydrogel network. Because of the resulting pendent thiols being hydrophilic, the disulfide cleavage increases hydrophilicity of hydrogel network. As a consequence, the resultant network exhibits enhanced thermoresponsiveness with a minor sacrifice in mechanical properties. The applicability of the thermoresponsive hydrogels toward biomedical applications was initially assessed with cell viability and enhanced release of encapsulated model hydrophilic drugs.

Experimental

Instrumentation and analysis

^{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. 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 refractive index (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^{-1}. Linear poly(methyl methacrylate) (PMMA) standards from Fluka were used for calibration. Aliquots of polymer samples were dissolved in DMF/LiBr. The clear solutions were filtered using a 0.25 μm PTFE filter to remove any DMF-insoluble species. A drop of anisole was added as a flow rate marker. Conversion was determined by ^{1}H-NMR for linear polymers and by gravimetry for hydrogels. UV/vis spectra were recorded on Agilent Cary 60 UV/vis spectrometer.

Materials

Poly(ethylene glycol) monomethyl ether (PEO–OH, MW = 5000 g mol^{-1} with ethylene oxide (EO)# ≈ 113), 2-bromoisoburylic acid (BriBuA, 98%), poly(ethylene glycol) methacrylate (POEOMA526, MW = 526 g mol^{-1} with EO# ≈ 10), 3,3′-dithiopropionic acid (ssDCOOH, 99%), *N*,*N*′-dicyclohexyl carbodiimide (DCC), *N*,*N*-dimethylaminopyridine (DMAP), Tin(II) 2-ethylhexanoate ($Sn(Oct)_2$, >95%), 2,2′-bipyridine (bpy, 98%), poly(ethylene glycol) dimethacrylate (DMA, M_w = 550 g mol^{-1}), Rhodamine-6G (R6G, 99%), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman reagent, ≥98%) from Aldrich Canada, copper(II) bromide ($CuBr_2$, >99.99%) from Acros Organics and dithiothreitol (DTT, ≥99%) from fisher scientific were purchased and used as received. Di(ethylene glycol) methyl ether methacrylate (MEO_2MA, 95%) from Aldrich was purified by passing it through a column filled with basic alumina to remove inhibitors. An aqueous buffer solution at pH = 8.0 was prepared by dissolving sodium phosphate dibasic (0.1 mM) and EDTA (1 mM) in deionized water.

PEO-functionalized 2-bromoisobutyrate (PEO–Br), a water-soluble ATRP macroinitiator, was synthesized as described elsewhere.[60] Briefly, PEO–OH (20 g, 4 mmol) reacted with BriBuA (740 mg, 4.4 mmol) in the presence of DCC (900 mg, 4.4 mmol) and a catalytic amount of DMAP in dichloromethane (DCM, 200 mL). The formed solids were removed by filtration under vacuum. The product was isolated *via* rotary evaporation of the solvent and further dried in a vacuum oven at 50 °C for 12 h ^{1}H-NMR ($CDCl_3$, 7.26 ppm): 1.9 (s, 6H, $-C(CH_3)_2Br$), 3.4 (s, 3H, $-OCH_3$), 3.45–3.80 (m, EO protons), 4.2 (m, 2H, $-CH_2-O(O)C-$).

Dithiopropionyl poly(ethylene glycol) dimethacrylate (ssDMA) was synthesized as described in literature.[61] Briefly, 3,3′-dithiopropionic acid (4.6 g, 20 mmol) in THF (60 mL) reacted with POEOMA526 (20 g, 38 mmol) in the presence of DCC (7.8 g, 38 mmol) and a catalytic amount of DMAP in DCM (120 mL). The product was purified by vacuum filtration and solvent evaporation. ^{1}H-NMR ($CDCl_3$, 7.26 ppm): 1.9 (s, 6H, $-CH_3$), 2.7 (*t*, 4H, $-C(O)-CH_2-CH_2-SS-$), 2.9 (*t*, 4H, $-C(O)-CH_2-CH_2-SS-$), 3.5–3.8 (m, PEO protons), 4.0–4.3 (m, 8H, $-C(O)O-CH_2-$ and $-CH_2-O(O)C-$), 5.6 (s, 2H, CH═), and 6.1 (s, 2H, CH═).

AGET ATRP of MEO_2MA in DMF

For kinetic studies, AGET ATRP of MeO_2MA in the presence of PEO–Br was carried out in DMF at 47 °C. MEO_2MA (5 g, 26.6 mmol), PEO–Br (272.8 mg, 0.053 mmol), $CuBr_2$ (35.6 mg, 0.16 mmol), bpy (49.8 mg, 0.32 mmol), and DMF (3.5 mL) were mixed in a 50 mL schlenk flask at room temperature. The resulting solution was stirred for 30 min, while being purged under nitrogen to remove oxygen. A nitrogen-purged anisole solution of $Sn(Oct)_2$ (375 mg mL^{-1}, 120 μL, 0.11 mmol) was added to the flask in order to reduce the Cu(II) complex to the activator Cu(I) complex and start the polymerization. Samples were withdrawn at different time intervals during the polymerization to determine conversion by NMR and provide molecular weight data by GPC. The polymerization was stopped by exposing the catalyst to air.

For purification, the as-synthesized solutions were diluted with acetone and then passed through a basic alumina column to remove residual copper. Acetone was removed by rotary evaporation at room temperature. The residues were precipitated from cold hexane three times and then dried under vacuum at room temperature for 18 h.

Synthesis of cleavable thermoresponsive hydrogel

The recipe optimized for the AGET ATRP of MEO_2MA in the presence of PEO–Br in DMF at 47 °C was used with various amounts of ssDMA crosslinker. Typically, to synthesize ssH2 gels in Table 1, MEO_2MA (5 g, 26.6 mmol), PEO–Br (272.8 mg, 0.053 mmol), $CuBr_2$ (35.6 mg, 0.16 mmol), bpy (49.8 mg, 0.32 mmol), ssDMA (2 mol% based on MEO_2MA, 290 mg, 0.53 mmol), and DMF (3.5 mL) were mixed in a 50 mL Schlenk flask at room temperature. Pieces of glass tubing (8 mm diameter and 2 cm length) were placed in the flask to act as cylindrical-shaped templates. The resulting solution was stirred for 30 min, while being purged under nitrogen to remove oxygen. A nitrogen-purged anisole solution of $Sn(Oct)_2$ (375 mg mL^{-1}, 120 μL, 0.11 mmol) was added to the flask in order to reduce the Cu(II) complex to the activator Cu(I) complex and start the polymerization. The polymerization was stopped at 8 h by exposing the catalyst to air.

The formed cylindrical hydrogels were purified as follows; the as-synthesized gels were immersed in fresh acetone (20 mL) five times for two days to remove remaining monomers and DMF solvent. They were then immersed in fresh deionized water to remove residual Cu species and acetone four times for two days. The purified gels were stored in water before use.

Determination of swelling ratio (SR)

Aliquots of swollen hydrogels were immersed in water at room temperature (RT) for 16 h. They were blotted against tissue paper to remove residual water (w_t, ≈100 mg). They were then dried at 120 °C for 4 h (w_d). The SR was determined by the weight ratio of swollen (w_t) to dried hydrogels (w_d).

Determination of LCST using Differential Scanning Calorimetry (DSC)

The hydrogel was swollen in water for 24 h. Aliquots of the swollen gels were then placed in a hermetic sample pan filled

with water (30 μL). Thermal properties of the gels were measured against a sealed reference pan filled with water (30 μL) with a TA Instruments DSC Q20 differential scanning calorimeter over a temperature range of 5 to 50 °C, at a heating rate of 2 °C min^{-1} from 5 to 50 °C under a nitrogen flow rate of 50 mL min^{-1}.

Deswelling kinetics by gravimetry

Water retention of hydrogels was measured as follows; aliquots of swollen hydrogels were placed in water at 45 °C for a given period time and weighed after being blotted against tissue paper to remove residual water (w_t). The procedure was repeated at specified time intervals over the course of 1 h. Water retention is calculated by $\frac{w_t - w_d}{w_0 - w_d}$, where w_t is the weight of swollen gels at time t, w_d is the weight of dried gels, and w_0 is the initial weight of swollen gels.

Reductive cleavage of disulfide linkages of ssH hydrogels

Aliquots of swollen hydrogels were immersed in an aqueous DTT solution under magnetic stirring at RT for 48 h. The degraded gels were extensively washed with water more than four times at 4 °C to remove excess DTT, yielding ssH-d gels.

Ellman assay for quantitative analysis of disulfide cleavages

A stock solution of DTNB at pH = 8 was prepared at 4 mg mL^{-1} concentration by dissolving DTNB (8 mg) in aqueous buffer solution (2 mL). Aliquots of the swollen, purified ssH-d gels were further washed with aqueous buffer solution (10 mL) more than five times to remove residual DTT. The complete removal of residual DTT in the final wash was monitored with DTNB. The further purified ssH-d gels were placed in fresh buffer solution (5 mL) and then mixed with aqueous DTNB stock solution (200 μL) at 4 °C for 1 h. The hydrogels were taken and washed with fresh buffer solution three times to remove residual NTB anions trapped inside the gels. The UV/vis spectra of the solutions were then recorded.

Viscoelastic measurements

Mechanical properties of ssH hydrogels were measured on a DHR-2 rheometer (TA Instruments, USA) in either temperature sweep mode or small amplitude oscillatory shear mode with a parallel plate geometry (8 mm diameter). For the temperature sweep mode, hydrogel samples dried in a vacuum oven at 60 °C for 24 h were loaded on the plates. The gap was set to obtain an axial force around 5 N, and temperature was varied in the range of −80 and 220 °C with a 1% strain at 1 rad s^{-1} frequency. For the oscillation mode, swollen hydrogels were loaded on the plates. The gap set to 2 mm and the oscillation frequency was varied in the range of 0.1–100 rad s^{-1} at a 1% strain.

Cell viability using MTT assay

HeLa cells were plated at 5×10^5 cells per well into a 96-well plate and incubated for 24 h in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (fetal bovine serum) and 1% antibiotics (50 units per mL penicillin and 50 units per mL streptomycin) at 37 °C in a humidified atmosphere containing 5% CO_2. They were then incubated with three pieces of each ssH4-d (21.7 ± 2 mg) and ssH4 (20 ± 1 mg) gels for 48 h. Prior to the incubation, gels were immersed in fresh PBS buffer (5 mL) three times for 12 h. Controls without gels (cells only) were run simultaneously. Cell viability was measured using CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega) according to manufacturer's instruction. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solutions (15 μL) was added into each well and after 4 h incubation the medium containing unreacted MTT was carefully removed. The formed blue formazan crystals were dissolved in stop solution (100 μL), the absorbance was measured at 570 nm using Powerwave HT Microplate Reader (Bio-Tek). Viability was calculated as the percent ratio of absorbance of mixtures with gels to control (cells only).

Loading and release of R6G using UV/vis spectroscopy

An aqueous stock solution of R6G at pH = 6 was prepared at a concentration of 0.17 mg mL^{-1}. The stock solution was diluted with different volumes of water, yielding a series of aqueous solutions with different concentrations of R6G. Their UV/vis spectra were recorded to determine its extinction coefficient at λ_{max} = 527 nm to be 79 800 M^{-1} cm^{-1}. Then, aliquots of hydrogels (30 mg in dried states) were immersed in the aqueous R6G stock solution (5 mL) at 4 °C for 48 h. After the R6G-loaded hydrogels were taken, UV/vis spectra of the solutions were recorded. The loading level of R6G was determined as the weight ratio of loaded R6G to dried gels. For the release of R6G from R6G-loaded hydrogels, the resulting R6G-loaded ssH and ssH-d gels were placed in a dialysis tubing with MWCO = 12 kDa and immersed in water at 45 °C. The absorbance was followed using a UV/vis spectrometer equipped with an external probe at 527 nm over time.

Results and discussion

Kinetic studies of AGET ATRP of MEO_2MA

Activators Generated by Electron Transfer (AGET) is an effective initiating process for atom transfer radical polymerization (ATRP).[59,61–63] AGET ATRP uses oxidatively stable Cu(II) complex which reacts with reducing agents such as $Sn(Oct)_2$ to generate active Cu(I) complex for normal initiation with the added alkyl halide initiator. In the experiments, DMF formed a homogeneous reaction mixture with $CuBr_2$/bpy complexes. A hydrophilic PEO-based bromine macroinitiator (PEO–Br) was synthesized by a facile carbodiimide coupling reaction of PEO–OH with BriBuA. AGET ATRP of MEO_2MA was initiated with PEO–Br in the presence of $CuBr_2$/bpy complexes in DMF at 47 °C. The conditions were $[MEO_2MA]_0/[PEO–Br]_0/[CuBr_2]_0/[bpy]_0$ = 500/1/3/6 with $[Sn(Oct)_2]_0/[CuBr_2]_0$ = 0.7/1 and MEO_2MA–DMF = 1.5/1 wt/wt.

Fig. S1† shows the kinetic results for synthesis of linear polymer chains. Polymerization was first-order, suggesting constant concentration of active centers during the

polymerization. An induction period of 1 h was observed. A similar induction period has been reported for AGET ATRP of 2-hydroxyethyl methacrylate (HEMA) with $CuBr_2$/bpy complex in a mixture of methyl ethyl ketone–MeOH (3/2 v/v) at 50 °C.[64] The occurrence of an induction period can be attributed to the slow reduction of Cu(II) to Cu(I) by $Sn(Oct)_2$ and the induction period decreased as the reaction temperature increased. Molecular weight increased linearly with conversion and molecular weight distribution was as narrow as $M_w/M_n < 1.2$ up to 60% conversion. These results suggest that polymerization proceeded in a living manner.

The resulting diblock copolymers were purified by removal of Cu species and unreacted monomers. GPC results show the evolution of GPC trace to a higher molecular weight region with no significant traces of PEO–Br macroinitiator remaining, thus yielding well-controlled PEO-*b*-$PMEO_2MA$ block copolymers (Fig. S2†).

Synthesis and characterization of cleavable thermoresponsive hydrogel precursors

The similar procedure as described above for AGET ATRP of MEO_2MA was applied to the synthesis of $PMEO_2MA$-based hydrogels crosslinked with disulfide linkages. A disulfide-labeled dimethacrylate (ssDMA) was synthesized using a facile carbodiimide coupling reaction. Various amounts of 2, 4, and 6 mol% ssDMA crosslinker were introduced into an AGET ATRP to synthesize well-defined disulfide-labeled cleavable thermoresponsive hydrogel precursors. Table 1 presents the characteristics of the cleavable hydrogels named as "ssH*x*", where *x* denotes the mol% of ssDMA in monomer mixtures.

Monomer conversion determined using gravimetry was close to 44–50%. Such low conversion suggests the presence of dangling chains of double bonds. Swelling ratio (SR) defined as the weight ratio of swollen to dried hydrogels in water was determined using gravimetry. The SR of ssH2 hydrogels was found to be as high as 10.2 and decreased to 3.6–3.8 (ssH4 and ssH6) when the amount of ssDMA increased to 4–6 mol% in monomer mixtures (Table 1). This is presumably attributed to the increasing crosslinking density as evidenced by the results of viscoelastic measurements of the dried hydrogels in the temperature sweep mode. Temperature was varied from −80 to 220 °C with 1% strain at 1 rad s^{-1} shear rate. Fig. S3† shows the viscoelastic properties of storage or elastic (G'), loss or viscous (G'') moduli, and tan δ (G''/G') over the full range of temperatures. A higher crosslinking density increases the elastic component of the gel with the increase in the storage modulus.

Table 1 Characteristics of thermoresponsive hydrogel precursors synthesized using AGET ATRP of MEO_2MA in the presence of cleavable ssDMA in DMF at 47 °C

Hydrogels	ssDMA/mol%	conv[a]	SR[a]	LCST[b] (C°)
ssH2	2	43.9 ± 6.8	10.2 ± 2.3	32.2
ssH4	4	48.4 ± 4.8	3.8 ± 1.1	32.3
ssH6	6	49.3 ± 3.9	3.6 ± 0.9	32.6

[a] Determined using gravimetry. [b] Determined using DSC.

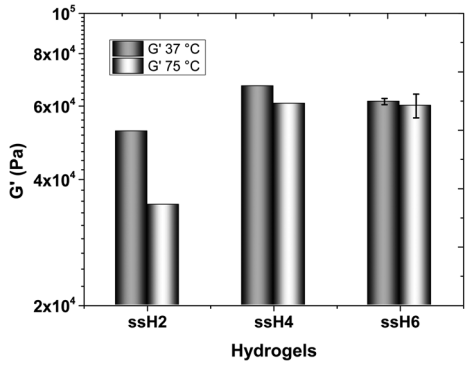


Fig. 1 Viscoelastic properties of storage modulus (G') at 37 °C and 75 °C of thermoresponsive ssH hydrogels. Note that the G' for ssH6 was averaged from three measurements with fresh gels.

Fig. 1 compares the G' values of the hydrogels at 37 °C (close to body temperature) and 75 °C, after they have reached a plateau after undergoing glass transition. At both temperatures, the G' value increased with an increasing amount of ssDMA (ssH2 *vs.* ssH4 hydrogels). The larger G' modulus is due to higher crosslinking densities of gel networks, which decreases the SR. Upon a further increase of ssDMA to 6 mol%, however, the G' value unchanged (ssH4 *vs.* ssH6 gels). Note that their SR is also similar. These results suggest that the amount of crosslinkers is an important parameter that influences the SR and mechanical properties of thermoresponsive hydrogels.

Thermoresponsive properties and deswelling kinetics

The thermoresponsive properties particularly lower critical solution temperature (LCST) of the resulting linear PEO-*b*-$PMEO_2MA$ block copolymer and disulfide-crosslinked hydrogels were examined. First, dynamic light scattering (DLS) was used to determined the linear PEO-*b*-$PMEO_2MA$. As seen in Fig. S4,† the normalized LS intensity increased sharply with an increasing temperature due to coil-globular transition by hydrophilic/hydrophobic transition upon heating. Its LCST determined from the onset of the increase in LS intensity was 33 °C. For disulfide-crosslinked hydrogels, DSC was used to determine their LCST,

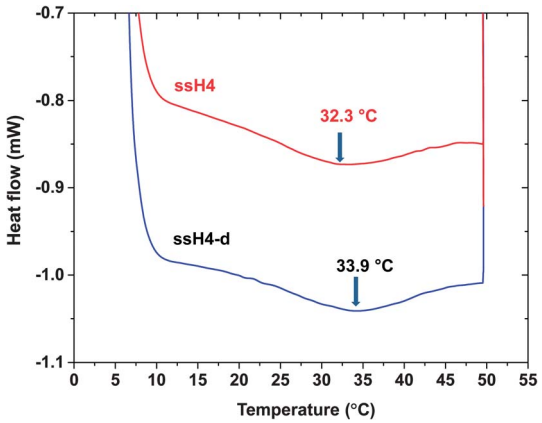


Fig. 2 As a typical example, DSC thermograms of ssH4 and ssH4-d hydrogels before and after treatment with excess DTT.

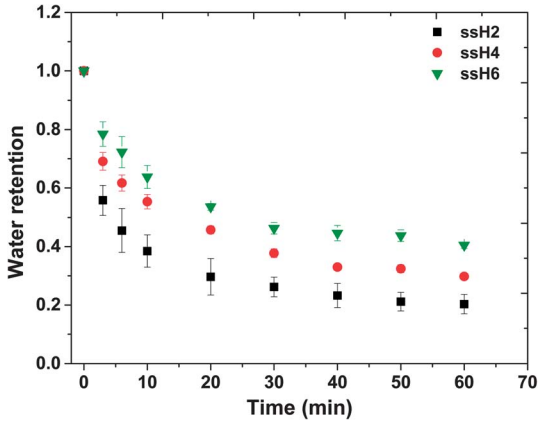


Fig. 3 Deswelling kinetics at 45 °C expressed by water retention for cleavable ssH hydrogels prepared with 2, 4, and 6 mol% ssDMA in water.

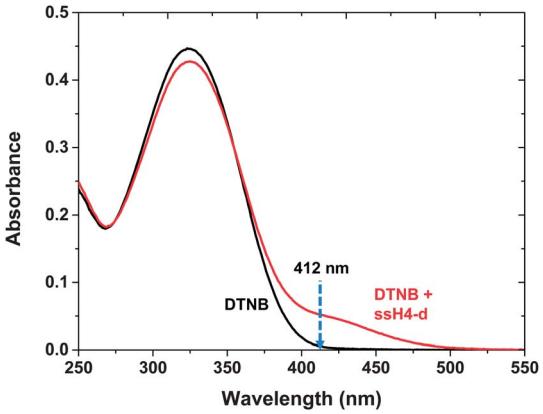


Fig. 4 UV spectra of DTNB and its mixture with ssH4-d in aqueous buffer solution at pH = 8.0.

as described for other hydrogels in the reports.[65,66] As seen in DSC thermogram of $PMEO_2MA$-based ssH4 hydrogels (Fig. 2) and other ssH2 and ssH4 gels (Fig. S5†), the temperature at the minimum point of the endotherm is defined as the LCST. As presented in Table 1, the LCST of all three hydrogels is ≈32 °C, close to that of their linear analog (PEO-*b*-$PMEO_2MA$).

The resulting cleavable thermoresponsive hydrogels undergo a volume change caused by a transition in hydrophobic/hydrophilic balance at the LCST. The rate of volume change (*i.e.* shrinking) of the hydrogels was examined by measuring the amount of water retained in hydrogels at a temperature of 45 °C, well above the LCST. The water retention is defined as the ratio of the amount of water retained in hydrogels at t to that at $t = 0$. As seen in Fig. 3, the water retention of ssH hydrogels decreased to reach a plateau over time. After 40 min, the water retention was 0.2 for ssH2, 0.3 for ssH4, and 0.45 for ssH6, suggesting slower shrinking kinetics with an increasing amounts of ssDMA crosslinker. The slow kinetics is presumably attributed to the increase in crosslinking densities of hydrogel networks.

Reductive cleavage of disulfides in ssH hydrogels and quantitative analysis

The resulting cleavable ssH hydrogels contain 2–6 mol% of disulfides in a crosslinked network. The disulfide linkages can be cleaved to the corresponding thiols in the presence of reducing agents such as D,L-dithiothreitol (DTT).[67,68] In the experiments, the purified ssH hydrogels were immersed in aqueous DTT solutions at room temperature for 2 days. The removal of excess DTT yielded degraded ssH gels (denoted as "ssH-d" gels). Note that ssH6 hydrogels were eliminated because they had similar properties, such as SR and viscoelastic properties to ssH4 hydrogels.

For the quantitative analysis of the cleavage of disulfide linkages with ssH2-d and ssH4-d gels, Ellman assay was used to determine the concentration of sulfhydryl (–SH) groups resulting from the cleavage of disulfide linkages. Ellman assay involves the spectroscopic determination of 2-nitro-5-thiobenzoate (NTB anion, yellow color) released from DTNB upon the cleavage of disulfide linkages by monitoring the reaction of DTNB with thiols in aqueous solutions. In the experiments, residual DTT was removed by extensive purification as DTT can also react with DTNB. As seen in Fig. 4, the UV/vis spectrum of DTNB exhibits a maximum absorption at λ = 326 nm. When ssH4-d gels were mixed with DTNB in aqueous solution, a new absorption peak at 400–500 nm appeared. The occurrence of the absorption is caused by the formation of NTB anions, indicating the cleavage of disulfide linkages in ssH-d gels in response to DTT. Using the Beer–Lambert equation with the absorbance and extinction coefficient at λ = 412 nm, the extent of cleavage of disulfides was determined to be 9.5% for ssH4-d gels and 19.4% for ssH2-d gels. At this point, it is not clear why not all disulfide linkages were cleaved in this system. The partial cleavage of disulfides results in ssH-d hydrogels which retain a solid shape and mechanical properties.

Analysis of degraded ssH-d gels

Because no significant cleavage of disulfide linkages is determined in ssH2 and ssH4 gels, the resulting ssH4-d gels were

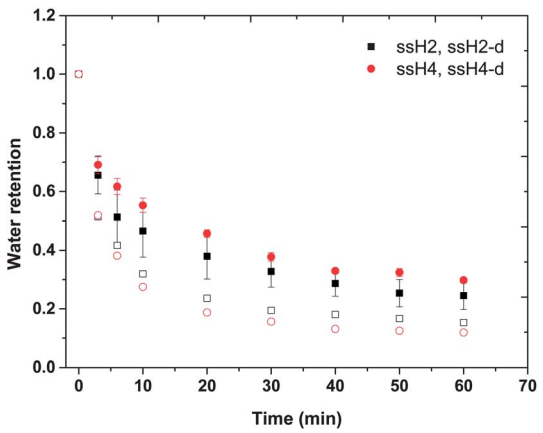


Fig. 5 Deswelling kinetics at 45 °C expressed by water retention of ssH-d gels (blank symbols), compared with ssH precursor gels (filled symbols).

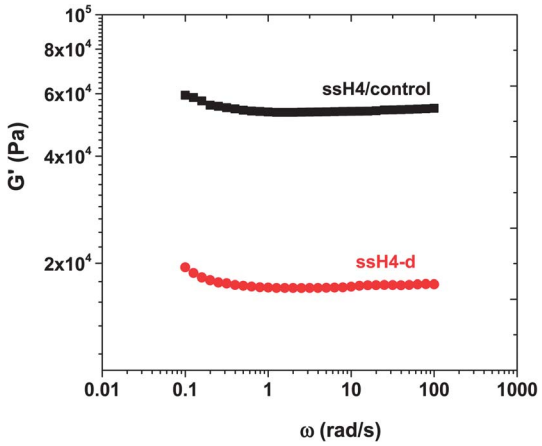


Fig. 6 Viscoelastic properties of G' modulus for wet ssH4-d gels, compared with wet ssH4 precursor gels in oscillatory shear mode.

typically characterized for LCST, deswelling kinetics, and viscoelstic properties. Their LCST using DSC was determined to be 33.9 °C (Fig. 2). This value is slightly higher than (32.3 °C) of ssH4 precursor gels; the difference is attributed to the increase in hydrophilicity of ssH gels to some extent as resulted from the formation of SH groups upon partial cleavage of disulfide linkages. Enhanced deswelling kinetics of ssH4-d gels, compared to ssH4 gels at 45 °C is shown in Fig. 5. The water retention after 40 min decreased to 0.2 for ssH4-d gels, significantly lower than 0.4 for ssH4 precursor gels. Such enhancement is also observed with ssH2 gels: the water retention = 0.2 for ssH2-d gels *vs.* 0.23 for ssH2 gels (Fig. 5). The enhanced thermoresponsiveness of ssH-d gels could be attributed to the generation of hydrophilic dangling chains with terminal SH groups and the increase in hydrophilicity of hydrogel network as resulted from the cleavage of disulfide linkages. Decreased mechanical properties of ssH gels after degradation are shown in Fig. 6. The G' modulus of wet ssH4-d gels were relatively lower than that of wet ssH4 gels in the oscillatory shear mode ranging from 0.01 to 100 rad s^{-1} at room temperature. The lower mechanical property of ssH4-d gels is attributed to the presence of partial cleavage of disulfide crosslinks to generate hydrophilic dangling chains in the hydrogels, thus increasing hydrophilicity of hydrogel network.

Biomedical perspectives

To preliminarily assess the ssH-d hydrogels toward biomedical applications, particularly drug delivery, *in vitro* cytotoxicity with HeLa cells were examined using MTT assay (a calorimetric method to measure cell toxicity). ssH4-d gels were cultured with HeLa cells, along with ssH4 gels for comparison and cells only as controls. After 48 h incubation, the absorbance was measured using absorbance-based plate reader and used to calculate cell viability. Fig. 7 shows >90% viability of HeLa cells in the presence of both ssH4 and ssH4-d gels, suggesting their non-cytotoxicity.

The release rate of rhodamine-6G (R6G) dye as a model hydrophilic drug was examined with ssH4-d gels, compared

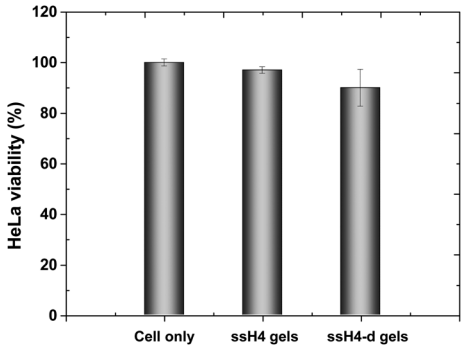


Fig. 7 Viability of HeLa cells cultured with ssH4 and ssH4-d gels for 48 h.

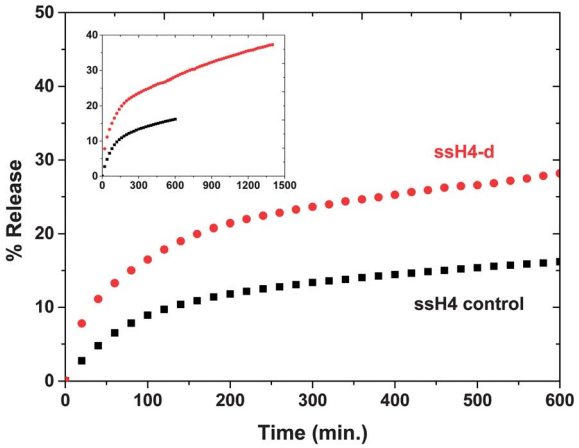


Fig. 8 Release of R6G as a model hydrophilic drug from R6G-loaded ssH4-d and ssH4 hydrogels in aqueous solution.

with their precursor of ssH4 gels. Using the extinction coefficient of R6G at λ_{max} = 527 nm in water (ε = 79 800 M^{-1} cm^{-1}, Fig. S6†), the loading level of R6G was determined to be 1.5% for ssH4-d gels, which is slightly higher than 1.3% for ssH4 gels. The release of R6G from these gels was examined using UV/vis spectrometer equipped with an external probe (Fig. S7†). Fig. 8 shows % release of R6G over time. For both gels, the rapid release was observed at the beginning; after 10 min, the release slowed down. The release reached 15% for ssH4 gels *vs.* 30% for ssH4-d gels in 600 min; however, it will reach the completed release over a longer time as suggested by the gradual release over time shown in the inset. Importantly, ssH4-d gels exhibit the enhanced release of R6G, compared to ssH4 gels. These results indicate that ssH-d gels exhibited higher loading and enhanced release of encapsulated hydrophilic model drugs.

Conclusion

Cleavable thermoresponsive hydrogels based on $PMEO_2MA$ crosslinked with disulfide linkages were prepared by crosslinking AGET ATRP of MEO_2MA and ssDMA in the presence of PEO–Br. In the absence of ssDMA, polymerization proceeded in a living fashion as evidenced with first-order kinetics, linear

increase of molecular weight with conversion, and narrow molecular weight distribution. Introduction of ssDMA allowed for the preparation of well-defined ssH precursor hydrogels exhibiting LCST $\approx$ 33 °C as determined by DSC. The properties of ssH hydrogels were varied with their network crosslinking densities; when the amount of ssDMA increased, SR and deswelling kinetics expressed by water retention decreased, while mechanical properties increased. Reductive cleavage of disulfide linkages of ssH gels yielded ssH-d gels with hydrophilic dangling chains with terminal SH groups. A 10–20% cleavage of disulfide linkages by quantitative analysis using Ellman assay increased LCST, enhanced deswelling kinetics, while slightly decreasing mechanical properties. With the balance of thermoresponsive and mechanical properties, combined with non-toxicity and enhanced release of encapsulated hydrophilic model drugs, ssH-d gels can find out their biomedical applications.

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