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Injectable, Degradable Thermoresponsive Poly(*N*-isopropylacrylamide) Hydrogels

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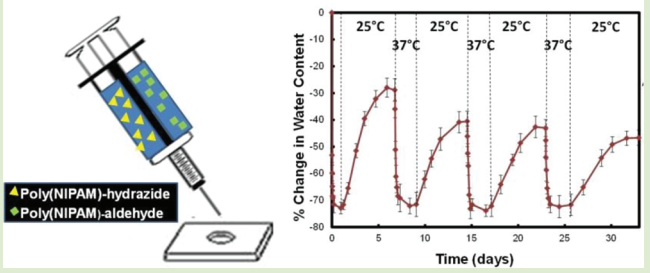
Supporting Information

ABSTRACT: Degradable, covalently in situ gelling analogues of thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAM) hydrogels have been designed by mixing aldehyde and hydrazide-functionalized PNIPAM oligomers with molecular weights below the renal cutoff. Co-extrusion of the reactive polymer solutions through a double-barreled syringe facilitates rapid gel formation within seconds. The resulting hydrazone cross-links hydrolytically degrade over several weeks into low molecular weight oligomers. The characteristic reversible thermoresponsive swelling−deswelling phase transition of PNIPAM hydrogels is demonstrated. Furthermore, both in vitro and in vivo toxicity assays indicated that the hydrogel as well as the precursor polymers/degradation products were nontoxic at biomedically relevant concentrations. This chemistry may thus represent a general approach for preparing covalently cross-linked, synthetic polymer hydrogels that are both injectable and degradable.



Thermoresponsive hydrogels based on poly(*N*-isopropylacrylamide) (PNIPAM) that switch from a hydrated, expanded state at low temperature to a collapsed state at high temperature have been extensively investigated in the literature. The proximity of the ~32 °C volume phase transition temperature (VPTT) of PNIPAM hydrogels with physiological temperature (37 °C) has sparked particular interest in the biomedical applications of these hydrogels as "smart", environmentally tunable drug delivery vehicles,[1] tissue engineering scaffolds,[2] cell growth/separation supports,[3] and biomolecule separation and recovery matrices,[4] among other applications.[5]

Despite their significant potential in biomedical applications, PNIPAM hydrogels have not achieved clinical success, primarily due to concerns regarding the ultimate fate of PNIPAM inside the body. Though toxic in its monomeric form,[5] poly(*N*-isopropylacrylamide) has been shown to be effectively noncytotoxic at concentrations realistic to many medical applications.[6] However, possible depolymerization and/or chronic bioaccumulation of PNIPAM represent significant regulatory barriers to medical use. An additional practical barrier is the need to fabricate current PNIPAM hydrogel formulations outside of the body, as the free radical chemistry and thermal or UV initiation[3] required to form the hydrogels can induce significant cell toxicity and cannot be performed in deep tissues. While weakly cross-linked hydrogels may be sufficiently viscous to facilitate injection,[4] injection of more highly elastic hydrogels is impractical. As a result, there is a need for mechanically robust PNIPAM-based hydrogels that can be introduced into the body through minimally invasive means and subsequently degrade into safe and clearable products.

While many studies have explored the formation of injectable and degradable thermoresponsive hydrogels via physical association[1,7,8] or space-filling,[9] relatively few studies have addressed the challenge of creating injectable and degradable covalently cross-linked PNIPAM hydrogels. Hydrolytically degradable hydrogels have been prepared by copolymerizing NIPAM with di(meth)acrylate cross-linkers containing a hydrolyzable internal segment, including poly(caprolactone),[10,11] poly(trimethylene carbonate),[12] acryloyloxyacryloyloxyethylaminopolysuccinimde,[13] ester linkages,[14] or chitosan.[15] Reducible hydrogels have been fabricated by incorporating disulfide linkages in the cross-linker.[16,17] Enzymatically degradable PNIPAM hydrogels have also been reported based on peptide cross-linkers.[18] In all these cases, chain transfer or controlled radical polymerization techniques can be used to limit the molecular weight of the polymers between degradable cross-linking points to promote clearance following degradation.[17] However, none of these formulations gel in situ upon injection, limiting the maximum mechanical strength of a hydrogel amenable for injection. In situ gelling natural polymer hydrogels have also been reported, using Michael addition,[19] Schiff base formation,[20] and hydrazone bond formation[21] to facilitate rapid gelation upon the mixing of reactive polymer precursors and functional cross-linkers. However, outside of hydrogels fabricated by mixing aldehyde and amine-functionalized Pluronic copolymers,[22] no covalently

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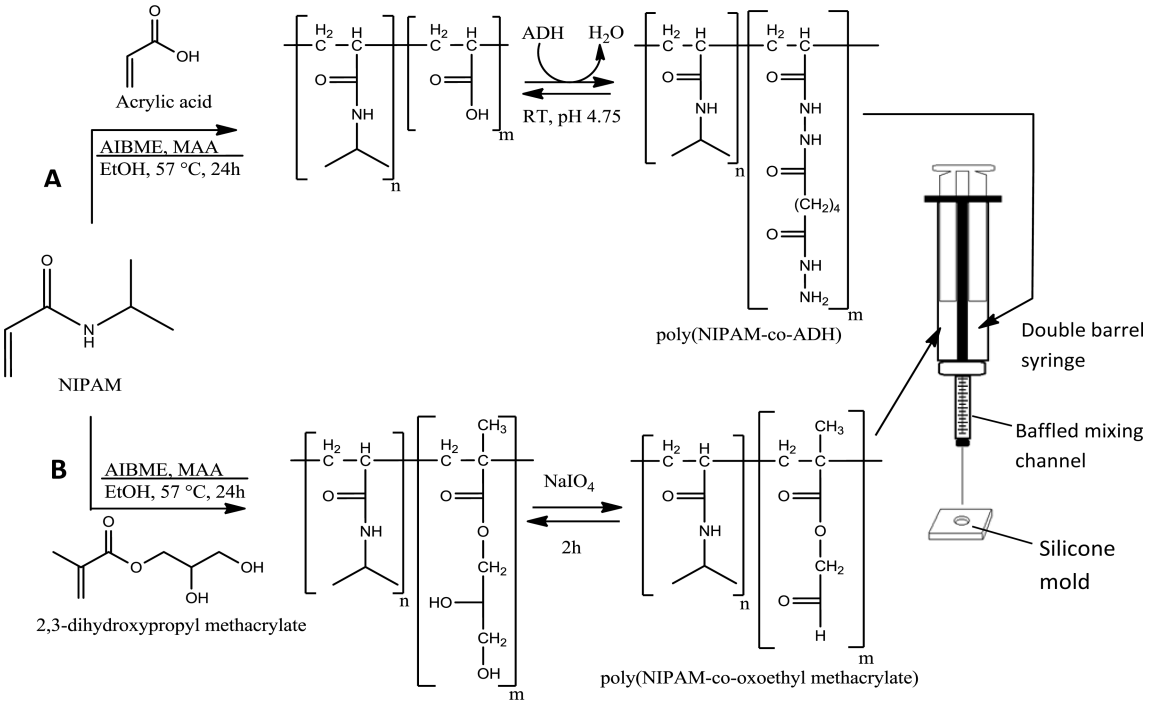

Figure 1. Synthesis of hydrazide-functionalized precursor copolymers (route A) and aldehyde-functionalized precursor copolymers (route B). Each precursor copolymer (dissolved at 6 wt % in 10 mM NaCl aqueous solutions) was added to its respective barrel of a double-barreled syringe. Precursors are then extruded along a mixing channel into a mold, where they rapidly cross-link to form the hydrogel.

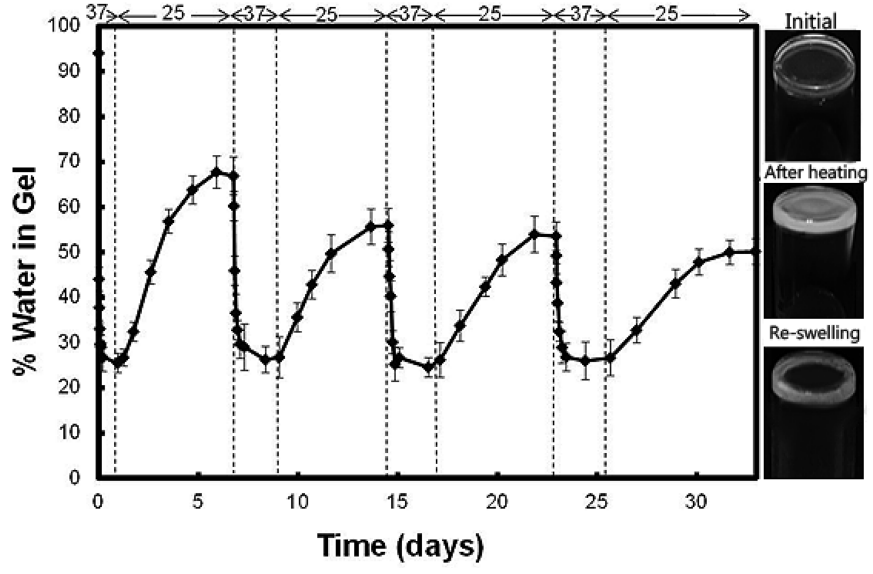

Figure 2. Reversible collapse/swelling of poly(NIPAM) hydrogel network incubated in 10 mM PBS (pH 7.4) at alternating temperatures of 25 and 37 °C (as noted at top of graph). Inset: Gel opacity resulting from a transition from the zero-strain state to 37 °C and reswelling at 25 °C.

cross-linked and in situ gellable thermoresponsive hydrogels have been reported.

In this communication, we report the design and synthesis of covalently cross-linked PNIPAM hydrogels that are both injectable and degradable based on PNIPAM copolymers possessing hydrazide and aldehyde functionalities. Figure 1 shows the chemistry used to synthesize the reactive polymeric precursors. Hydrazide-functionalized polymer precursors (poly(NIPAM-*co*-ADH)) were synthesized by functionalizing poly(NIPAM-*co*-acrylic acid) polymers with adipic acid dihydrazide via carbodiimide chemistry. Aldehyde-functionalized polymer precursors (poly(NIPAM-*co*-oxoethyl methacrylate) were synthesized via copolymerization of NIPAM with dihydroxypropyl methacrylate and subsequent oxidation of the diol. In both cases, use of a chain transfer agent limits the molecular weight of the polymers (M_n < 22 kDa) below the renal cutoff (~40 kDa), facilitating potential clearance of the degradation products from the body. To make a hydrogel, reactive polymers were individually dissolved in 6 wt % 10 mM NaCl aqueous solutions, loaded into separate barrels of a double-barreled syringe, and coextruded through a turbulent mixer into a silicone mold. Formation of the hydrogel network occurred in

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less than one minute following coextrusion. This rapid gelation would permit the formation of a gel network before the low-viscosity linear polymer precursors are able to diffuse away from their site of introduction, a significant problem with slower-reacting in situ gelation chemistries.

The reversible thermal response of the resulting PNIPAM hydrogel is demonstrated in Figure 2. Hydrogels were incubated in 10 mM PBS (pH 7.4) and were alternately placed on shaking platforms maintained at 25 and 37 °C for defined time intervals to assay the reversibility of the PNIPAM hydrogel phase transition.

The initial collapse of the hydrogel network occurs more rapidly (5.1 h) than subsequent cycles (7.7 h on average), while reswelling of the network takes place over a number of days before the equilibrium swelling condition is again reached. Although the hydrogels do not fully recover to the zero strain state following their first incubation at 37 °C, hydrogels exhibit reversible swelling−deswelling transitions upon subsequent cycles; this is consistent with previous reports of conventional PNIPAM hydrogels.[9] The swelling response is mirrored by changes in the turbidity of the hydrogel, which switches from nearly transparent immediately following gel formation to completely opaque upon thermal collapse to semitranslucent upon rehydration (Figure 2, side panel). The relatively long time period required for full reswelling at each cycle likely results from the high degree of cross-linking between precursor copolymers. Based on the degree of functionalization of the constituent polymers (14.3 ± 0.3% hydrazide groups and 11 ± 3% aldehyde groups), the average number of monomers between cross-links N_x is as high as ∼12 in these injectable hydrogels, equivalent to the preparation of conventional hydrogels with ∼8.5 mol % cross-linker; conventional PNIPAM hydrogels of similar cross-link densities have been reported to require several days to reswell to their equilibrium state upon a temperature change.[23] Thus, the injectable hydrogels exhibit the same thermal deswelling and scattering properties of conventional, nondegradable PNIPAM hydrogels.

Figure 3 demonstrates the degradability of the synthesized PNIPAM hydrogels in response to acid-catalyzed hydrolysis at

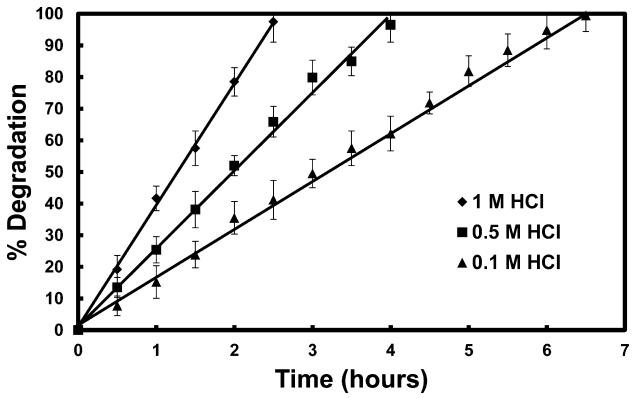

Figure 3. Degradation of PNIPAM hydrogels on incubation at 37 °C in 0.1 M, 0.5 M, and 1 M HCl at constant ionic strength.

constant [H^+] concentrations (see Supporting Information, Figure S1-A). The increase in degradation rate with increasing acid concentration demonstrates that bulk loss of hydrogel occurs by proton-catalyzed hydrolysis of the hydrazone cross-links (the carbon−carbon polymer backbone is not hydrolytically susceptible). It should also be noted that minimal hydrolysis of amide groups in PNIPAM residues has been observed using the same catalytic conditions.[24] Extrapolation of the measured kinetics using a power law correlation that fits the data predicts a gel lifetime of several months at physiological pH (pH = 7.4), consistent with previously reported hydrolytic lifetimes of hydrazone cross-links.[25] GPC analysis of degradation products demonstrates that the gels degrade back to precursor copolymers (see Supporting Information, Figure S1-B). Note that the maximum water content (measured gravimetrically) upon thermal cycling decreases (albeit slightly) upon multiple cycles (p = 0.048, Figure 2), indicating a loss of hydrogel mass as a function of time that may be related to slow degradation of the hydrogel in the PBS media used for release studies. By degrading the hydrogels at defined chemical cross-linking points, we expect that the toxic effects observed with monomeric byproduct may be avoided[6] without compromising the potential clearance of the polymer degradation products through the kidney. The time frame over which this degradation occurs may be tunable by altering the number of cross-links between the polymer precursors, by reducing the amount of oxoethyl methacrylate and acrylic acid (i.e., conjugation sites for adipic acid dihydrazide) in the base polymer used to synthesize the hydrogels, reducing the degree of oxoethyl methacrylate oxidation, or by reducing the molecular weight of the PNIPAM gel precursors.

In vitro and in vivo toxicity studies of the injectable PNIPAM hydrogels and the hydrazide and aldehyde-reactive precursor copolymers are shown in Figure 4. In vitro cell viability assays were performed using an MTT assay with 3T3 mouse fibroblasts and retinal pigment epithelial (RPE) cells. Note that retinal pigment epithelium cells are grown in serum-free media, permitting cytotoxicity assessments both in response to the pure material (RPE cells) and a protein-adsorbed material (3T3 cells). Poly(NIPAM-*co*-ADH) induces a slight cytotoxic response at concentrations greater than 400 μg/mL in 3T3 cells (Figure 4A) and no significant toxicity at any tested concentration in RPE cells (Figure 4B). Poly(NIPAM-*co*-oxoethyl methacrylate) induces only mild cytotoxicity to both cell types at low-to-moderate concentrations but becomes more cytotoxic at very high concentrations (i.e., 2000 μg/mL, Figures 4A,B). Overall, any observed cytotoxicity occurs at polymer concentrations well above any local concentrations expected due to the slow hydrolytic degradation of hydrogel under physiological conditions. Similarly, 3T3 cells incubated in the presence of a PNIPAM hydrogel showed no significant decrease in cell viability relative to a cell-only control (viability = 0.99 ± 0.11 relative to a cell-only control).

Acute (48 h postinjection) in vivo toxicity assays were performed via subcutaneous injection of BALB/c mice with 0.35 mL of 6 wt % solutions of PNIPAM polymer precursors (Figures 4-C and D), and a 6 wt % in situ-formed hydrogel (Figure 4-E). Hematoxylin-eosin (H&E) staining indicates that the reactive copolymer solutions are largely cleared from the injection site 48 h postinjection and induce only a very mild acute inflammatory response, similar to that observed with the PBS control injection (see Supporting Information, Figure S2). The in situ-gelled PNIPAM hydrogel remains at the injection site after 48 h and induces a mild inflammatory response at the tissue−hydrogel interface consisting predominantly of macrophages with a few neutrophils. However, the adjacent muscle tissue maintains its polygonal, multinucleate structure and blood vessels remained unoccluded. Following a five-month chronic incubation of the gel in vivo (Figure 4F) it is observed

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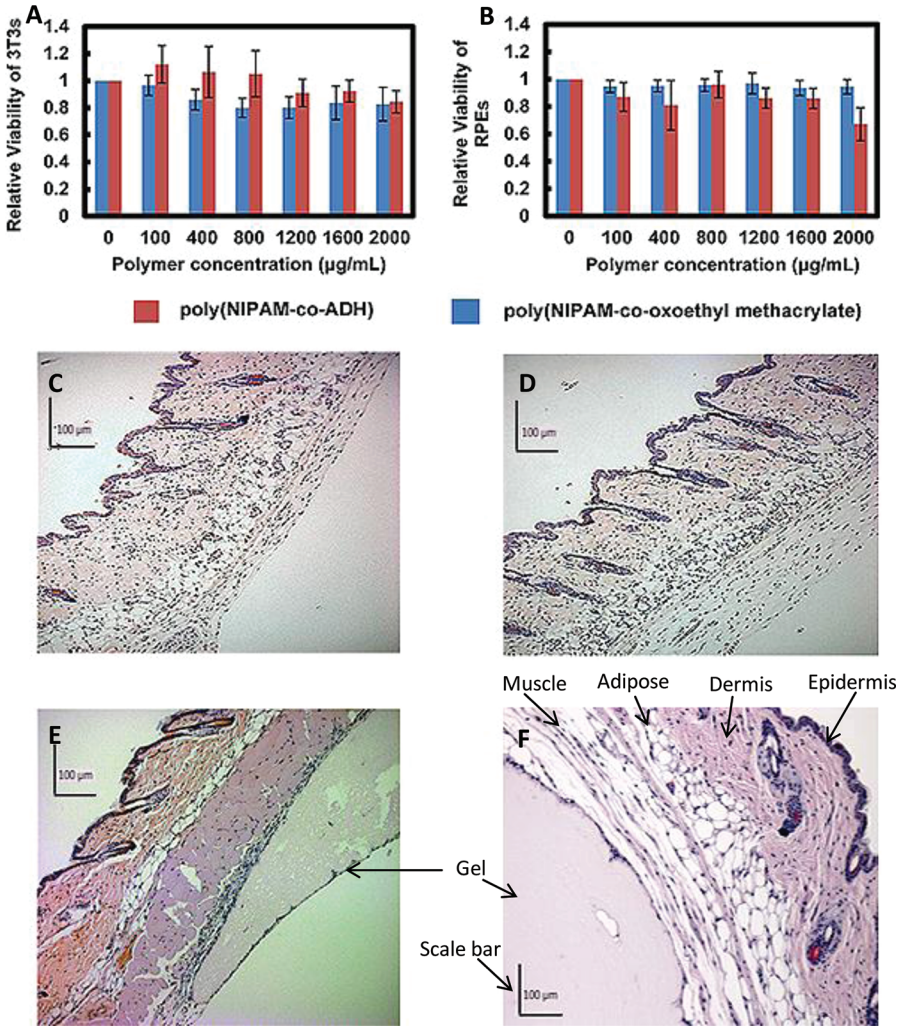

Figure 4. Cytotoxicity and biocompatibility of injectable hydrogels and precursor/degradation product polymers: (A) MTT viability assays of 3T3 mouse fibroblasts in the presence of both precursor copolymers at various concentrations; (B) MTT viability assays of RPE retinal pigment epithelial cells in the presence of both precursor copolymers at various concentrations; (C–F) H&E stained sections of mouse subcutaneous tissue: (C) 6 wt % poly(NIPAM-*co*-ADH) in PBS, after 48 h; (D) 6 wt % poly(NIPAM-*co*-oxoethyl methacrylate) in PBS, after 48 h; (E) PNIPAM in situ-formed hydrogel from 6 wt % polymer precursor solutions in PBS, after 48 h; (F) PNIPAM in situ-formed hydrogel from 6 wt % polymer precursor solutions in PBS, after five months. Tissue labels on (F) are pertinent to all histological samples.

that the gel remains at the site of introduction, although the quantity of residual gel was somewhat lower than the initial injected volume. However, minimal to no fibrous capsule formation was observed at the gel–tissue interface and no sign of a chronic inflammatory response was noted either within the residual gel or within the tissue adjacent to the gel relative to the PBS-only control (Supporting Information, Figure S2). Together, these results suggest that the gel is well-tolerated within the subcutaneous space and may be amenable for practical use in in vivo applications.

In summary, we have described a novel method to synthesize covalently cross-linked and in situ-gellable thermoresponsive hydrogels based on poly(N-isopropylacrylamide) using reversible and rapid hydrazide-aldehyde chemistry to link functionalized PNIPAM oligomers. The hydrogels exhibit the same thermal swelling–deswelling responses as conventional PNIPAM hydrogels but can be degraded back into the reactive polymer gel precursors via an acid-catalyzed hydrolysis process. Furthermore, the combination of rapid gelation with low toxicity observed herein suggests that this hydrazide-aldehyde oligomer cross-linking approach may be translatable to the design of injectable, degradable covalently cross-linked hydrogels based on a range of biocompatible synthetic polymers.

■ EXPERIMENTAL METHODS

Synthesis of poly(NIPAM-*co*-ADH): Hydrazide-functionalized polymers were synthesized via adipic acid dihydrazide functionalization of acid-functionalized PNIPAM.[10] Poly(NIPAM-*co*-acrylic acid) polymers were first synthesized by reacting *N*-isopropylacrylamide monomer (4.5 g), acrylic acid (0.5 g), 2,2-azobisisobutyric acid dimethyl ester (AIBME) initiator (0.056 g), and thioglycolic acid chain transfer agent (80 μL) in 20 mL of absolute ethanol overnight at 56 °C under nitrogen. Solvent was then removed under reduced pressure and the resulting viscous product was dialyzed over six cycles (12–14 kDa MWCO) against distilled deionized water. Gel permeation chromatography (GPC) indicated the product polymer had a molecular weight of M_n = 21.6 kDa (PDI 1.65), below the renal cutoff. Conductometric titration indicated that the copolymers contained 14.5 ± 0.3 mol % of acrylic acid. Hydrazide groups were then grafted to the acrylic acid residues by dissolving 0.5 g of poly(NIPAM-*co*-AA) and 10.15 g of adipic acid dihydrazide (ADH) together with 5.58 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC) in 100 mL of water adjusted to pH 4.75. The pH was maintained throughout the

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reaction by dropwise addition of 0.1 M HCl until no further pH change was observed (5 h total). The resulting solution was exhaustively dialyzed for three days (12−14 kDa MWCO) against distilled deionized water. Conductometric titration showed a 99% consumption of acrylic acid groups, resulting in a 14.3 ± 0.3 mol % functionalization of polymer with ADH. ^{1}H NMR (Bruker 200 mHz) in DMSO: δ 6.50−7.80 (1.45H); 4.75−5.25 (1H); 10.82 (10.82H).

Synthesis of poly(NIPAM-*co*-oxoethyl methacrylate): *N*-Isopropylacrylamide monomer (4.5 g), 2,3-dihydroxypropyl methacrylate (DHOPMA, 0.5 g), AIBME initiator (0.056 g), and thioglycolic acid chain transfer agent (80 μL) were dissolved in 20 mL of absolute ethanol and reacted overnight at 56 °C under nitrogen. The resulting copolymer was isolated in a similar manner to that described above. ^{1}H NMR analysis indicated that the copolymer contained 10 mol % DHOPMA. The *cis*-diol groups present in the purified copolymer were then oxidized by dissolving 1 g of the diol-containing copolymer and 1.5 g sodium periodate in 150 mL H_2O for 2 h, with 200 μL of ethylene glycol then added to stop the reaction. The oxidized copolymer was purified in the same manner as above. GPC analysis indicated M_n = 15100 kDa (PDI 1.51), below the renal cutoff. A *tert*-butyl carbazate-based aldehyde detection assay[26] indicated that 11 ± 3 mol % of total monomer residues in the polymer contained aldehyde groups. This result was confirmed by ^{1}H NMR, which gave a value of 10.5 mol % of aldehyde groups within a polymer chain. ^{1}H NMR (Bruker 200 mHz) in DMSO: δ 9.45−9.6 (0.08H); 6.75−7.75 (1H); 5.25−4.75 (0.16H); 3.5−4.0 (1H); 0.0−2.25 (9.4H).

Hydrogel synthesis and testing: Solutions [6 wt % (m/v)] of each precursor copolymer in 10 mM NaCl were added to separate barrels of a double barrel syringe and coextruded into a 0.37 mL circular silicone mold (diameter 6 mm and height 2 mm) through a turbulent mixer to create hydrogel samples for testing (Figure 1). Thermosensitivity assays were conducted by loading the hydrogel samples (n = 6) inside cell culture inserts (2.5 cm, 8 μm pore size) placed in 12-well cell culture plates containing 2 mL of 10 mM phosphate buffered saline (PBS, total ionic strength 0.15M) at pH 7.4. Gels were incubated in alternating cycles of 37 °C (above VPTT) and 25 °C (below VPTT) until equilibrium was reached. The change in gel mass (related directly to the water content of the gel) was determined gravimetrically at each time point indicated in Figure 2. Degradation assays were conducted by loading the hydrogel samples (n = 6) into the same cell culture inserts, which are subsequently placed in wells containing 0.1, 0.5, or 1 M hydrochloric acid (HCl). Changes in gel mass were measured gravimetrically every 30 min until the bulk gels were completely degraded.

Toxicity assays: Cytotoxicity in the presence of various concentrations (0−2 mg/mL) of reactive polymers and hydrogels was tested in vitro in 24 h cycles, using 3T3 *Mus musculus* mouse cells and human retinal pigment epithelial (RPE) cells as model cells. A MTT assay was used to quantify cell viability, following published methods.[12] In vivo toxicity was assayed using a subcutaneous injection model, injecting n = 4 BALB/c mice (22−24 g, Charles River Laboratories) with 0.35 mL total of (a) 6 wt % solutions of reactive polymer precursors dissolved in 0.15 M NaCl; (b) a 6 wt % PNIPAM hydrogel prepared by mixing 6 wt % hydrazide and aldehyde-functionalized polymers (both dissolved in 0.15 M NaCl) inside a double-barreled syringe (Medimix, Switzerland); and (c) phosphate buffered saline as a control. Animals were visually observed to identify any toxic response, with two animals sacrificed 2 days following injection (acute response) and another two animals sacrificed 150 days after injection (chronic response). A tissue sample including skin, underlying tissue, and (if present) residual polymer was recovered from the animals and subjected to histological analysis using hematoxylin and eosin staining. Animals were cared for in compliance with protocols approved by the Animal Research Ethics Board at McMaster University and regulations of the Animals for Research Act of the Province of Ontario and the guidelines of the Canadian Council on Animal Care.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental details and source materials are described. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Moon, H. J.; Choi, B. G.; Park, M. H.; Joo, M. K.; Jeong, B. *Biomacromolecules* **2011**, *12*, 1234−1242.
(2) Stile, R. A.; Burghardt, W. R.; Healy, K. E. *Macromolecules* **1999**, *32*, 7370−7379.
(3) Yang, J.; Yamato, M.; Shimizu, T.; Sekine, H.; Ohashi, K.; Kanzaki, M.; Ohki, T.; Nishida, K.; Okano, T. *Biomaterials* **2007**, *28*, 5033−5043.
(4) Kim, J.; Park, K. *Bioseparation* **1998**, *7*, 177−184.
(5) Schild, H. G. *Prog. Polym. Sci.* **1992**, *17*, 163−249.
(6) Malonne, H.; Eeckman, F.; Fontaine, D.; Otto, A.; Vos, L. D.; Moes, A.; Fontaine, J.; Amighi, K. *Eur. J. Pharm. Biopharm.* **2005**, *61*, 188−94.
(7) Ho, E.; Lowman, A.; Marcolongo, M. *Biomacromolecules* **2006**, *7*, 3223−3228.
(8) Lee, B. H.; Vernon, B. *Macromol. Biosci.* **2005**, *5*, 629−635.
(9) Garbern, J. C.; Hoffman, A. S.; Stayton, P. S. *Biomacromolecules* **2010**, *11*, 1833−1839.
(10) Galperin, A.; Long, T. J.; Ratner, B. D. *Biomacromolecules* **2010**, *11*, 2583−2592.
(11) París, R.; Quijada-Garrido, I. *Polym. Int.* **2009**, *58*, 362−367.
(12) Wang, F.; Li, Z.; Khan, M.; Tamama, K.; Kuppusamy, P.; Wagner, W. R.; Sen, C. K.; Guan, J. *Acta Biomater.* **2010**, *6*, 1978−1991.
(13) Yu, Y. Q.; Li, Z. Z.; Tian, H. J..; Zhang, S. S..; Ouyang, P. K. *Colloid Polym. Sci.* **2007**, *285*, 1553−1560.
(14) Siegwart, D. J.; Bencherif, S. A.; Srinivasan, A.; Hollinger, J. O.; Matyjaszewski, K. *J. Biomed. Mater. Res. A* **2008**, *87A*, 345−358.
(15) Yu, Y.; Chang, X.; Ning, H.; Zhang, S. *Cent. Eur. J. Chem.* **2008**, *6*, 107−113.
(16) Tsarevsky, N. V. *Macromolecules* **2005**, *38*, 3087−3092.
(17) Zhang, B. Y.; He, W. D.; Li, L. Y.; Sun, X. L.; Li, W. T.; Zhang, K. R.. *J. Polym. Sci., Part A: Polym. Chem.* **2010**, *48*, 3604−3612.
(18) Kim, S.; Healy, K. E. *Biomacromolecules* **2003**, *4*, 1214−1223.
(19) Hiemstra, C.; van der Aa, L. J.; Zhong, Z.; Dijkstra, P. J.; Feijen, J. *Biomacromolecules* **2007**, *8*, 1548−1556.
(20) Nishi, K. K.; Jayakrishnan, A. *Biomacromolecules* **2007**, *8*, 84−90.
(21) Yeo, Y.; Ito, T.; Bellas, E.; Highley, C. B.; Marini, R.; Kohane, D. S. *Ann. Surg.* **2007**, *245*, 819−824.
(22) Nam, J. A.; Al-Nahain, A.; Hong, S.; Lee, K. D.; Lee, H.; Park, S. Y. *Macromol. Biosci.* **2011**, DOI: 10.1002/mabi.201100265.
(23) Xiao, X. C. *eXPRESS Polym. Lett.* **2007**, *1*, 232−235.
(24) Hoare, T.; Pelton, R. *Langmuir* **2004**, *20*, 2123−2133.
(25) Vetrík, M.; Přádný, M.; Hrubý, M.; Michálek, J. *Polym. Degrad. Stab.* **2011**, *96*, 756−759.
(26) Maia, J.; Ferreira, L.; Carvalho, R.; Ramos, M. A.; Gil, M. H. *Polymer* **2005**, *46*, 9604−9614.