Synthesis, Physicochemical Characterization, and Cytocompatibility of Bioresorbable, Dual-Gelling Injectable Hydrogels

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Supporting Information

ABSTRACT: Injectable, dual-gelling hydrogels were successfully developed through the combination of physical thermogellation at 37 °C and favorable amine:epoxy chemical cross-linking. Poly(N-isopropylacrylamide)-based thermogelling macromers with a hydrolyzable lactone ring and epoxy pendant groups and a biodegradable diamine-functionalized polyamidoamine cross-linker were synthesized, characterized, and combined to produce nonsyneresing and bioresorbable hydrogels. Differential scanning calorimetry and oscillatory rheometry demonstrated the rapid and dual-gelling nature of the hydrogel formation. The postgelation dimensional stability, swelling, and mechanical behavior of the hydrogel system were shown to be easily tuned in the synthesis and formulation stages. The leachable products were found to be cytocompatible under all conditions, while the degradation products demonstrated a dose- and time-dependent response due to solution osmolality. Preliminary encapsulation studies showed mesenchymal stem cell viability could be maintained for 7 days. The results suggest that injectable and thermally and chemically cross-linkable hydrogels are promising alternatives to prefabricated biomaterials for tissue engineering applications, particularly for cell delivery.

1. INTRODUCTION

Injectable, in situ forming hydrogels are a promising alternative to implantable prefabricated scaffolds investigated for craniofacial tissue engineering. Because of their ability to form 3-D, highly hydrated substrates in a minimally invasive manner, this versatile class of polymer-based biomaterials can enable local delivery of growth factors and cells, can easily create scaffolds that fill and conform to complex configurations, and can provide a supportive environment for cell migration and proliferation. The benefits and approaches toward designing injectable hydrogels for tissue engineering have been recently reviewed.[1–6]

Thermoresponsive polymers are particularly attractive injectable materials because gelation to a physically cross-linked hydrogel is triggered solely by temperature elevation to and above the lower critical solution temperature (LCST). Hydrogels based on one polymer, poly(N-isopropylacrylamide) (PNiPAAm), have been widely investigated for various biomedical applications, as it undergoes a sol–gel transition at its LCST of 32 °C.[7] Previous work has determined various means to tune and modulate the gelation kinetics[8] and has demonstrated controlled environmentally responsive drug and growth factor delivery.[9,10] Additionally, PNiPAAm-based hydrogels have been successfully used for the encapsulation of articular chondrocytes in vitro.[11] However, the main challenges associated with PNiPAAm-based hydrogels have been syneresis and nondegradability, limiting their efficacy for tissue engineering applications. Syneresis, or shrinkage of the hydrogel over time, can reduce nutrient diffusion to encapsulated cells. Additionally, hydrogel shrinkage would minimize hydrogel-tissue contact within the defect, impeding tissue integration. Biodegradation is also important, as nondegradable materials present a physical barrier to further tissue repair and increase the chances of an inflammatory response. Thus, rational design of PNiPAAm hydrogels to address both factors can drastically advance the properties of these materials for controlled delivery and improved integration in tissue engineering applications.[12]

A number of groups have attempted to enhance bioresorbability of PNiPAAm polymers by copolymerizing complex pendant groups such as lactate ester,[13,14] or polyester[15] side groups, 2-methylene-1,3-dioxepane,[16] poly(amino acid),[17] or 2-
hydroxyethylmethacrylate-6-hydroxyhexanoate\textsuperscript{18} that modulate the LCST over time. The materials became increasingly resorbable when the LCST of the polymer rose sufficiently above body temperature. In these cases involving polyesters, lactate side chains, or poly(AMAs), the monomers also produced soluble degradation products that may be toxic to encapsulated cells and raise the pH of the local microenvironment. In a different study, Cui et al. achieved PNiPAAm biodegradation without toxic byproducts through incorporation of dimethyl-γ-butyrolactone acrylate (DBA), a hydrolyzable lactone ring, allowing for the modulation of the LCST hypothesized that the copolymerization of NiPAAm, a LCST between room and physiologic temperature. We of both the GMA and DBA comonomers and tuned the initial addition of hydrophilic AA compensated for the hydrophobicity resolubilize over time via LCST modulation. Lastly, the lyzable lactone ring, which would enable the polymer to cross-linker. Time-dependent degradation of hydrogel would be pendant epoxy rings with the amine groups of the PAMAM biodegradation without toxic byproducts through incorporation of analytical columns (Waters Styragel guard column 20 mm, 4.6 × 30 mm; Waters ultrahydrogel column 1000, 7.8 × 300 mm) was used to determine the molecular weight distributions of the synthesized TGMs.\textsuperscript{23} The TGM was first hydrolyzed under accelerated conditions (see Before and After Hydrolysis LCST section) to remove its thermogelling properties. The weight-average molecular weight ($M_w$), number-average molecular weight ($M_n$), and polydispersity index (PDI = $M_w / M_n$) of the hydrolyzed polymer were determined by comparison with commercially available narrowly dispersed molecular weight poly(ethylene glycol) (PEG) standards (Waters, Mississauga, ON).  

2. MATERIALS AND METHODS

2.1. Materials. NiPAAm, DBA, GMA, AA, 2,2′-azobis(2-methylpropionitrile) (azobisisobutyronitrile, AIBN), N,N′-methylene-bisacrylamide (MBA), and piperazine (PIP) were purchased from Sigma Aldrich (Sigma, St. Louis, MO) and used as received. Anhydrous 1,4-dioxane, diethyl ether, and acetone in analytical grade; water, acetonitrile, chloroform, and methanol in HPLC-grade; and 1 N sodium hydroxide (NaOH) were purchased from VWR (Radnor, PA) and used as received. PBS (powder, pH 7.4) was obtained from Gibco Life, Grand Island, NY. Ultrapure water was obtained from a Millipore Super-Q water system (Millipore, Billerica, MA).

2.2. TGM Synthesis and Characterization. Synthesis of P(NiPAAm-co-GMA-co-DBA-co-AA) TGM was performed according to established protocols,\textsuperscript{19,22} as shown in Scheme 1. In a typical reaction, 10 g of NiPAAm, GMA, DBA, and AA was dissolved in anhydrous 1,4-dioxane under nitrogen at 65 °C. AIBN in dioxane was added at 0.7% of total mol content to initiate free radical polymerization, and the reaction mixture was stirred for 16 h. After solvent removal by rotary evaporation, the material was redissolved in acetone and purified twice via dropwise precipitation in excess diethyl ether. The recovered polymer was air-dried overnight and transferred to a vacuum oven for several days prior to elemental analysis. The chemical composition of the TGMs was determined by proton nuclear magnetic resonance spectroscopy (400 MHz °H NMR, Bruker, Switzerland). The polymer was dissolved in D$_2$O at a concentration of 20 mg/mL that contained 0.75 wt % 3-(trimethylsilyl)propionic-2,2,3,3-d$_4$ acid and sodium salt as an internal shift reference (Sigma-Aldrich, St. Louis, MO), and the data were analyzed using the MestReC NMR software package (Mestrelab Research S.L., Spain) and quantitative NMR analysis. Acid titration was performed in conjunction with °H NMR to determine the AA content of the TGMs before hydrolysis. Aqueous gel permeation chromatography (GPC) using a Waters Alliance HPLC system (Milford, MA), and differential refractometer (Waters, model 410) equipped with a series of analytical columns (Waters Styragel guard column 20 mm, 4.6 × 30 mm; Waters ultrahydrogel column 1000, 7.8 × 300 mm) was used to determine the molecular weight distributions of the synthesized TGMs.\textsuperscript{21} The TGM was first hydrolyzed under accelerated conditions (see Before and After Hydrolysis LCST section) to remove its thermogelling properties. The weight-average molecular weight ($M_w$), number-average molecular weight ($M_n$), and polydispersity index (PDI = $M_w / M_n$) of the hydrolyzed polymer were determined by comparison with commercially available narrowly dispersed molecular weight poly(ethylene glycol) (PEG) standards (Waters, Mississauga, ON).

Scheme 1. Synthesis of P(NiPAAm-co-GMA-co-DBA-co-AA) Thermogelling Macromer (TGM) via Radical Copolymerization of N-Isopropylacrylamide (NiPAAm), Glycidyl Methacrylate (GMA), Dimethyl-γ-butyrolactone acrylate (DBA), and Acrylic Acid (AA)
2.3. PAMAM Synthesis and Characterization. PAMAM was synthesized by the polyaddition of PIP and MBA at a stoichiometric ratio of [MBA]/[PIP] = 0.75 or 0.85 following previously reported protocols. Molecular weight distributions of the synthesized PAMAM cross-linkers were analyzed using time-of-flight mass spectrometry with positive-mode electrospray ionization on a Bruker microTOF ESI spectrometer (Bruker Daltonics, Billerica, MA) equipped with a 1200 series HPLC (Agilent Technologies, Santa Clara, CA) to deliver the mobile phase (50:50 HPLC-grade water and methanol). After data acquisition, all peaks (including degradation and secondary reaction products) were identified using microTOF Control software (Bruker) following published procedures. The peaks were corrected for charge state (generally with H+ or Na+ and rarely with K+ ions) and quantified for calculation of $M_n$, $M_w$, and PDI.

2.4. Before and After Hydrolysis LCST. The LCSTs of the TGMs before and after hydrolysis were determined by differential scanning calorimetry (DSC). Unhydrolyzed and hydrolyzed polymers were dissolved in PBS pH 7.4 to create 10 wt % solutions. Hydrolyzed polymers were created by dissolving TGM in ultrapure water or PBS pH 7.4 at 4 °C in 4 mL glass vials, followed by the addition of 50 μL of 0.1 N NaOH solution. The solutions were dialyzed against water and were used immediately for DSC.

Fourteen μL of each polymer solution was pipetted into an aluminum foil sample pan (TA Instruments, Newcastle, DE) and capped/crimped. Thermograms were recorded on a TA Instruments DSC 2920 equipped with a refrigerated cooling system against an empty sealed pan as reference. The oven was equilibrated at 5 °C and then heated to 80 °C at a heating rate of 5 °C/min. The LCST was determined both as the onset and peak temperature of the endothermic peak in the thermogram using the Universal Analysis 2000 software provided with the DSC system.

2.5. Hydrogel Fabrication. Hydrogels (6 mm in diameter, 3 mm in height) were fabricated by combining the TGM and PAMAM crosslinker. Individual solutions of TGM and PAMAM cross-linker were prepared at twice the desired concentrations in PBS pH 7.4 and placed on a shaker table at 4 °C until dissolved. The PAMAM solution was pipetted into the TGM solution using cold pipet tips, and the resulting solution was manually mixed in the glass vial. 90 μL injections were transferred to 6 mm diameter × 3 mm height cylindrical Teflon molds at 37 °C and allowed to gel for 24 h. To evaluate the macroscopic properties of the hydrogel system, we fabricated the hydrogels as described in the above section, except that after manual mixing the glass vial was transferred to a water bath at 37 °C for immediate observation of the thermogelling properties. Syneresis was qualitatively evaluated after 24 h at 37°C.

2.6. Rheological Characterization. A rheostated, oscillating rheometer (RheoLab AR1000, TA Instruments, New Castle, DE) equipped with a 6 cm steel cone (1°) with a gap size of 26 μm at a frequency of 1 Hz and displacement of 1 × 10^-4 rad was used to evaluate the elastic response of the hydrogels. Hydrogel formulations in pH 7.4 PBS were pipetted onto the rheometer, and the dynamic viscoelastic properties of the solutions, namely, the dynamic shear storage (G') and loss (G'') modulus, complex viscosity (η*), and loss angle (δ), were recorded using the TA Rheology Advantage software (TA Instruments). The solution was maintained at 4 °C, followed by elevation to and maintenance at 37 °C for 3 h to monitor the crosslinking reaction.

2.7. In Vitro Degradation. Accelerated degradation studies were performed in accordance with ISO 10993 standards. Hydrogels were fabricated in 6 mm diameter × 3 mm height cylindrical Teflon molds. Hydrogels were weighed at formation ($W_0$) and then frozen and lyophilized. After initial dry weight was measured ($W_i$), gels were reswollen in 4 mL glass vials with 4 mL of PBS pH 10.5 at 70 °C, and weekly solution changes were performed. At each time point, the samples (n = 6) were carefully blotted, weighed ($W_{c}$), and lyophilized. The dry weight was measured ($W_d$), and the percent of polymer loss was calculated through the difference between the initial and final dry weights divided by the initial dry mass ($W_i - W_d/ W_i * 100$). The leachables and degradation products were analyzed using liquid chromatography with positive-mode electrospray ionization on a Bruker maXis 4G HR TOF spectrometer equipped with a 1200 series HPLC (Agilent Technologies, Santa Clara, CA) to deliver the mobile phase (50:50 HPLC-grade water and methanol). After data acquisition, all peaks were identified using microTOF Control software (Bruker) following published procedures. The peaks were corrected for charge state (generally with H+ or Na+ and rarely with K+ ions) and quantified for calculation of $M_n$, $M_w$, and PDI.

2.8. Hydrogel Swelling. Hydrogel swelling behavior was assessed in a 24 factorial experimental design with formulations of varying TGM wt %, DBA mol content, PAMAM molecular weight, and amine:epoxy mol ratio. The individual solutions were fabricated as previously described in 6 mm diameter × 3 mm height cylindrical molds at 37 °C. After 24 h, the hydrogels were weighed on a balance and placed in excess PBS for 24 h at 37 °C. Hydrogels were then weighed after swelling, frozen at ~80 °C, lyophilized, and reweighed when dry. The hydrogel swelling, ratio formation ($R_{formation}$) and equilibrium ($R_{equilibrium}$) was calculated as the difference between the swollen and dry mass divided by the dry mass.

2.9. Mechanical Testing. Hydrogel formulations with varying TGM wt % and amine:epoxy mol ratio (n = 6 per group) were fabricated as previously described. Mechanical testing on a TA Instruments Thermomechanical Analyzer 2940 (TA Instruments, Newcastle, DE) equipped with a wide compression probe (diameter of 6 mm) was performed to assess the unconfined compressive Young's modulus of the hydrogels. Samples were first placed onto the prewarmed stage, and sample height was measured by the probe. The stage was then re-equilibrated to 37 °C and the sample was compressed at a rate of 0.001 N/min to 0.05 N. The unconfined Young's modulus was determined to be the initial slope of the engineering strain versus engineering stress curve.

2.10. Cytocompatibility Testing. 2.10.1. Cell Culture. A rat fibroblast cell line (ATCC, CRL-1764) was chosen for the in vitro cytocompatibility tests. The cells were cultured on T-75 flasks using Dulbecco’s modified Eagle’s medium (DMEM; Gibco Life) supplemented with 10% (v/v) fetal bovine serum (FBS; Cambrex BioSciences, Walkersville, MD) and 1% (v/v) antibiotics containing penicillin, streptomycin, and amphotericin (Gibco Life). Cells were cultured in a humidified incubator at 37 °C and 5% CO2. Cells of passage numbers 3–5 were used.

2.10.2. In Vitro Cytocompatibility. The leachables and degradation product assays for cytocompatibility were performed following established protocols. Solutions of hydrogels were prepared at the appropriate concentrations by dissolving TGM and PAMAM in serum-free DMEM at 4 °C. The hydrogels were fabricated in 6 mm diameter × 3 mm height Teflon molds and allowed to gel for 24 h in a 37 °C incubator. Hydrogels were then processed for the leachables or degradation products assay, and the test media were diluted to 1:10, 10:1, and 100 times original concentration. Cultured fibroblasts were passaged at ~80–90% confluence, and 96-well plates were seeded at a cell density of 10,000 cells/well and cultured for 24–48 h until 90% confluent. The test media and its dilutions were fed at 100 μL/well to the cells (n = 6), replacing the original media, and incubated for two established time points, 2 or 4 h, when at 37 °C, 95% relative humidity, and 5% CO2. Cells fed serum-free media (without polymer) served as a positive (live) control, and cells exposed to 70% ethanol for 10 min were used as a negative (dead) control.

After incubation, the test media were removed and the cells were rinsed three times with pH 7.4 PBS before adding calcein AM and ethidium homodimer-1 at 2 and 4 μM concentrations in PBS, respectively (Live/Dead viability/cytotoxicity kit, Molecular Probes, Eugene, OR). The cells were incubated in the dark at room temperature for 30 min. Cell viability was quantified using a fluorescence plate reader (Biotek Instrument FLx800, Winoomoki, VT) equipped with filter sets of 485/528 nm (excitation/emission) for calcein AM (live cells) and 285/620 nm (excitation/emission) for EthD-1 (dead cells). The fluorescence of the cell populations was recorded, and the fractions of live and dead cells were calculated relative to the controls.

2.10.3. Leachables Assay. For the leachables assay, hydrogels were incubated in serum-free media for 24 h at a 37 °C warm room at a 3 cm²/mL surface area/volume ratio according to established procedures. The test media were then sterile-filtered and incubated with the cells.

2.10.4. Degradation Products Assay. For the degradation products study, hydrogels were placed in ultrapure water with 2 mL of 1 N NaOH solution at 37 °C until fully degraded. Dialysis was not performed on the solution, as the degradation products may range in molecular weight. The solution with the complete degradation products was frozen at ~80 °C, lyophilized, reconstituted into the solution, and then used for the in vitro cytocompatibility testing.

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same volume of serum-free media required for fabrication, and sterile-filtered prior to cell exchange.

2.10.5. Ion Concentration Cytocompatibility. Media with 0, 30, 60, 90, 120, and 220 mg NaCl/10 mL DMEM were measured via osmometry, sterile-filtered, and incubated with cells, and their viability was recorded at 24 h (n = 6) to isolate the effects of solution osmolality from the degradation products. NaCl was chosen as a biocompatible molecule that would allow for isolation of the effects of osmolality without introducing any effects of pH or molecular composition on cell viability. The quantity of NaCl added was calculated to be the theoretical amount of NaCl salt to raise a 10 mL media solution by 100 mOsm.

2.10.6. Osmolality Determination. Solution osmolality was determined by measurement with an Osmette A automatic osmometer (Precision Systems, Natick, MA) calibrated with 100 and 500 mOsm/kg H2O standard solutions (Precision Systems) and run on the 0–2k mOsm range.

2.11. MSC Encapsulation. An encapsulation study was performed to demonstrate the cell-delivery capabilities of the hydrogel. MSCs were harvested from rat femora and tibiae of 6–8 week old Fisher 344 rats (Charles River Laboratories, Wilmington, MA) following established procedures in accordance with approved protocols by the Rice Institutional Animal Care and Use Committee. The rats were euthanized by CO2 asphyxiation and a bilateral thoracotomy following anesthesia using 4% isoFlurane/O2 mixture (Baxter Healthcare, Deerfield, IL). The tibia and femora were aseptically removed, placed in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) (Cambrex Healthcare, Deerfield, IL). The tibiae and femora were asceptically placed in a sterile media solution by 100 mOsm/ff osmolality without introducing any eects of pH or molecular composition on cell viability.

6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0

Figure 1. 1H NMR spectrum of P(NiPAAm-co-GMA-co-DBA-co-AA) TGM. Peak proton locations are identified with letters a–l.

and the remaining polymer backbone protons are located from 1.3 to 2.3 ppm. The relative locations of the peaks correlate similarly to those of PNiPAAm homopolymer, GMA monomer (data not shown), and similar PNiPAAm-DBA copolymers published in the literature. Calculations of the DBA mol content using the relative intensities of the DBA 2h protons gave an average value of 5.8 ± 0.3 and 2.5 ± 0.3%. The Mw/Mn and PDI of the 6% DBA-containing TGM were found to be 56 ± 4600 Da, 113 600 ± 2500 Da, and 2.02 ± 0.12, respectively, with the 2.5% DBA-containing TGM at similar values.

The before and after hydrolysis LCSTs of the TGMs were determined by DSC. The thermal properties of the synthesized TGM formulations are presented in Table 1. Copolymerization of NiPAAm with GMA and 6 or 2.5% DBA led to an initial peak LCST of 22.4 ± 1.0 or 27.7 ± 0.1 °C, respectively. This initial peak LCST is lower than the reported LCST of the PNiPAAm homopolymer (32 °C) due to the hydrophobicity of the GMA and DBA comonomers. However, after hydrolysis of the ester groups in the ring to hydroxyl and carboxyl groups, the higher DBA content leads to an increase in hydrophilicity, which is associated with a higher LCST. Therefore, the 6% DBA TGM has a higher hydrolysis LCST of 63.3 ± 3.7 °C compared with 39.5 ± 3.6 °C for the 2.5% DBA TGM. The initial LCSTs determined by DSC corresponded to the macroscopically observed thermogulation of the macromers and the reported hydrolysis of DBA in the literature.

3. RESULTS AND DISCUSSION

3.1. TGM Synthesis and Characterization. Copolymers of NiPAAm with reactive functional moieties for chemical cross-linking and LCST modulation were successfully synthesized. The chemical composition of the TGMs was characterized by NMR. The 1H NMR spectra of P(NiPAAm-co-GMA-co-DBA-co-AA) TGM are shown in Figure 1. The five pendant protons on GMA are found between 2.8 and 4.8 ppm, along with the isopropyl proton on NiPAAm (3.9 ppm). The three protons on DBA are found at the double peak at 5.6 and 5.8 ppm and the single peak at 4.2 ppm, correlating to the relative peaks of the DBA comonomer (data not shown). The six NiPAAm methyl protons were located from 0.9 to 1.3 ppm.
postformation syneresis. Upon mixing of the TGM and PAMAM solutions at 4 °C and transfer to 37 °C, the mixture began to thermogel instantaneously, as indicated by the formation of an opaque white hydrogel. After a period of 30 min, the sol–gel transition was accompanied by a second color change in which the hydrogel became translucent. The cross-linking reaction was completed within 3 h, and, as hypothesized, a stable, nonshrinking hydrogel was observed compared with hydrogels formed by the TGM alone (Figure 2). Distinct rheological traces of the hydrogel cross-linking in the absence of thermogelation by the maintenance of the hydrogel nature of the hydrogels (Figure 3). The 3 h run was the presence of thermogelation clearly demonstrate the dual gelling nature of the hydrogels (Figure 3). The 3 h run was stopped at 155 min after the adhesiveness of the hydrogel after further oscillation and accurate measurement. A rapid increase in the shear storage and loss moduli was observed in the initial 5 min of the trace, correlating to thermogelation at 37 °C, followed by slower chemical cross-linking, which resulted in an ultimate shear storage modulus of 100 kPa. The storage modulus of the gel without chemical cross-linking peaks at ~100 Pa at 37 °C; however, the thermodynamic instability of the hydrogels in the absence of chemical gelation results in a decreased storage modulus and complex viscosity in the subsequent 15–30 min at 37 °C, which is observed in other studies.8

The slight decrease in the first 15 min of the rheological trace is believed to be a result of the rapid loss in the thermogelling properties of the hydrogels due to a preferential hydrolysis of the DBA lactone ring at neutral and basic pH (data not shown). During the mixing of the TGM and PAMAM solutions, the PAMAM solution, which is basic, likely accelerated the hydrolysis of the DBA comonomer, leading to a loss in thermogelation at a faster rate than observed in previous studies.59 This rapid change in LCST, while unexpected, simplified the degradation rate to be dependent only on PAMAM hydrolysis, which has been explored previously.21,22 Chemical gelation of the epoxy and amine groups in the absence of thermogelation was also performed via rheology similar to published studies.22 While the interaction between the carboxylic acid groups of the TGM with the PAMAM cross-linkers is likely, as shown by other studies involving acids and amines,29 we hypothesize that due to the greater amount of amine and epoxy units and favorability of reaction,30,31 the epoxy-amine reaction will dominate the cross-linking process. However, it was not possible to directly measure the crossover of the shear loss and shear storage moduli under the same conditions using rheology because the LCST of this system was lower, requiring a lower controlled temperature that would alter the hydrogel’s original water content and cross-linking kinetics. Chemical cross-linking was macroscopically observed in the absence of thermogelation by the maintenance of the hydrogel shape after transfer to a 4 °C environment (Supplementary Figure 1 in the Supporting Information).

3.4. Accelerated Degradation. In vitro degradation studies were performed using 10 wt % hydrogels with 6% DBA and 1440 Da PAMAM at a 1:1 amine:epoxy ratio under basic accelerated conditions (PBS pH 10.5 at 70 °C). Degradation was calculated as percent dry polymer mass loss over time. Figure 4 shows that the hydrogels fully degraded under accelerated conditions over 30 days. The 30% weight loss after 1 day is attributed to the loss of sol fraction after incubation in PBS because gels were fabricated in confined molds without excess water. Hydrolytic degradation of the PAMAM amide bonds and DBA ester groups was accelerated over time. Figure 4 shows that the hydrogels fully degraded under accelerated conditions over 30 days.

Table 2. Characterization of Synthesized PAMAM Crosslinkers

<table>
<thead>
<tr>
<th>PAMAM stoichiometric feed ratio (r)</th>
<th>Mw (Da)</th>
<th>Mn (Da)</th>
<th>PDI</th>
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<tbody>
<tr>
<td>p-1440</td>
<td>0.78</td>
<td>1450</td>
<td>1.38</td>
</tr>
<tr>
<td>p-2600</td>
<td>0.85</td>
<td>2580</td>
<td>1.31</td>
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Table 1. TGM Synthesis and Thermal Characteristics

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<tr>
<th>TGM</th>
<th>PNiPAAm</th>
<th>GMA</th>
<th>DBA</th>
<th>AA</th>
<th>PNiPAAm</th>
<th>GMA</th>
<th>DBA</th>
<th>AA</th>
<th>initial peak LCST (°C)</th>
<th>hydrolysis peak LCST (°C)</th>
</tr>
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<tbody>
<tr>
<td>DBA-6</td>
<td>80.5</td>
<td>7.5</td>
<td>7</td>
<td>5</td>
<td>84.9 ± 0.06</td>
<td>9.1 ± 0.35</td>
<td>5.8 ± 0.26</td>
<td>3.2 ± 0.32</td>
<td>22.4 ± 1.0</td>
<td>63.3 ± 3.7</td>
</tr>
<tr>
<td>DBA-2.5</td>
<td>86.5</td>
<td>7.5</td>
<td>3.5</td>
<td>2.5</td>
<td>86.5 ± 0.20</td>
<td>7.0 ± 0.51</td>
<td>2.5 ± 0.28</td>
<td>3.5 ± 0.36</td>
<td>27.7 ± 0.1</td>
<td>39.5 ± 3.6</td>
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Figure 2. Thermogelation of 10 wt % TGM containing 6 mol % DBA with (a) and without (b) addition of P-1440 PAMAM after 1 min at 37 °C.

Figure 3. Oscillatory rheology trace showing shear storage (G”) and loss (G’) moduli for a 10 wt % hydrogel composition containing 6 mol % DBA cross-linked with P-1440 PAMAM at 1:1 amine:epoxy ratio held at 4 °C for 1 min and then 37 °C for 3 h.
3.5. Hydrogel Swelling. The effects of different hydrogel parameters on the degree of postformation syneresis and hydrogel stability were examined in a factorial swelling study. The parameters varied were the TGM polymer amount in the injectable solution (TGM wt %), functionality ratio between amine groups of the PAMAM and epoxy groups on the TGM (amine:epoxy mol ratio), the DBA mol content, and the molecular weight (MW) of the PAMAM cross-linker. It should be noted that the 1:1 amine:epoxy mol ratio corresponds to the theoretical maximum degree of cross-linking for each combination of TGM and PAMAM macromers studied.

The weight swelling ratio ($q$) of the hydrogels was measured at formation (24 h after fabrication in molds, $q_f$) and equilibrium (24 h after swelling in PBS at 37 °C, $q_s$). Figure 5 shows the swelling capacity of the hydrogels in the factorial study. For all hydrogel formulations, formation swelling ratios for the 15 and 20 wt % groups were lower than those of the 10 wt % groups due to the higher polymer content. At equilibrium, the weight swelling ratios increased proportionately. The higher TGM wt % groups resisted further expansion due to their more tightly cross-linked network. Greater equilibrium swelling was also observed in the P-2600-containing formulations as well as the higher amine:epoxy ratio, indicating that the PAMAM MW and greater incorporation of branches contributed to the overall hydrophilicity and the subsequent swelling behavior.

To more extensively evaluate the effects and interactions of the four parameters (TGM wt %, amine:epoxy mol ratio, DBA mol content, and PAMAM MW) on hydrogel swelling behavior, we performed a full $2^4$ factorial analysis. Table 3 lists the high/low values of each parameter.

The results were analyzed with SAS JMP v.10 software and are illustrated in Figure 6, which shows the main effects of parameters at formation and equilibrium with error bars representing the standard error of each effect population. The zero line represents the overall population mean for each effect (5.20 ± 0.38 and 6.93 ± 0.37 for formation and equilibrium, respectively). TGM wt % (factor c), or polymer content of TGM, was the most significant factor at formation, the effect of which is maintained at equilibrium. Higher TGM wt % decreased swelling through reduced availability of water in the injected solution and a tightly cross-linked network that was

\begin{figure}
\centering
\includegraphics[width=\textwidth]{degradation_profile.png}
\caption{Degradation profile of a 10 wt % hydrogel with 6 mol % DBA cross-linked with P-1440 PAMAM at 1:1 amine:epoxy ratio under accelerated conditions (pH 10.5 at 70 °C). Data are reported as means ± standard deviation for a sample size of $n = 4$.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{swelling_ratio.png}
\caption{Formation and equilibrium weight swelling ratio of 36 different hydrogel formulations (6 mm in diameter, 3 mm in height) in a factorial study. Hydrogels vary in DBA mol content (2.5 or 6%), PAMAM molecular weight (1440 or 2600 Da), amine:epoxy mol ratio (0.75:1, 1:1, or 1.25:1), and TGM wt % (10, 15, or 20 wt %) in PBS pH 7.4 at 37 °C. Data are reported as means ± standard deviation for a sample size of $n = 4–6$.}
\end{figure}
resistant to further expansion. The other factors displayed a positive significant impact only at equilibrium, with increases in the PAMAM MW (factor b), DBA mol content (factor a), and amine:epoxy mol ratio (factor d) leading to greater equilibrium swelling by 0.56 ± 0.39, 0.94 ± 0.39, and 0.76 ± 0.39, respectively. All three parameters resulted in an increase in overall gel hydrophilicity due to higher total mol content of PAMAM (amine:epoxy mol ratio), higher PAMAM mass (PAMAM MW), or exposure of hydrophilic groups (DBA mol content). The DBA mol content was hypothesized to have a lesser effect than the other factors on formation and equilibrium swelling; however, the expedited degradation of the DBA on a rapid time scale to produce hydrophilic hydroxyl and carboxyl groups increased polymer hydrophilicity and contributed to its unexpected greater impact at equilibrium.

A number of significant cross interactions are also observed in the formation and equilibrium weight swelling ratios, which are illustrated in Figure 7. Although increasing DBA mol content only led to a significant main effect in equilibrium swelling, this effect was enhanced by lower PAMAM MW at both formation and equilibrium (ab interaction). The higher swelling due to the increased gel hydrophilicity from the accelerated time-dependent hydrolysis of the DBA ring was likely initially offset for the lower PAMAM MW cross-linked hydrogels, which, due to their shorter cross-link interchain bridge length, reduced their capacity for subsequent expansion.32,33 This cross effect between high DBA mol content and lower PAMAM MW was additionally observed at formation with the lower TGM wt % (abc interaction). It was likely that the higher overall water content and greater expansion potential of the 10 wt %, P-2600 hydrogels coupled to the contribution of hydrophilic groups from hydrolyzed DBA led to a transient increased swelling response. At equilibrium, interaction effects were observed between higher final hydrophilicity and a more loosely cross-linked network (ac interaction) due to the higher DBA mol content and lower TGM wt %. Finally, the cd interaction at formation between the PAMAM mol content (amine:epoxy ratio) and the TGM wt % was primarily a reflection of the reduced initial water content for the same volume of injected solution.

### 3.6 Mechanical Testing

Another factorial study was performed to examine the tunability of the mechanical behavior of the hydrogels. Unconfined compression testing was performed on 6 mm diameter × 3 mm height hydrogels with varying TGM wt % (10, 15, 20 wt %) and amine:epoxy mol ratio (0.5:1, 1:1). Using 6% DBA and 1440 Da PAMAM hydrogels, all of the groups displayed an unconfined compressive modulus, \(E\), in the kilopascal range, with the lowest modulus for the 10 wt % 0.5:1 cross-linked group at 1.72 kPa and highest modulus for the 15 wt % 1:1 cross-linked group at 7.26 kPa (Figure 8). The results indicate that higher TGM wt % (15 and 20 wt %) and higher cross-linking density led to significant increases in the Young’s modulus. No significant differences were found between the different groups when the cross-linking density was halved (0.5:1 amine:epoxy mol ratio). The higher TGM wt % groups possessed a greater polymer density at the maximum cross-linking ratio, allowing for an increased compressive strength. However, at the lower amine:epoxy ratio, there was little dimensional stability, and the gels performed similarly under the compression test. Additionally, although the 15 wt % 1:1 cross-linking hydrogels...
demonstrated the highest modulus, there was not a significant difference between this group and the 20 wt % 1:1 cross-linking group.

3.7. Cytocompatibility Testing. The cytocompatibility of the hydrogel system was evaluated by treating rat fibroblast cells in media with 1, 10, and 100× dilutions of leachable products from the different TGM and cross-linked hydrogel formulations or the degradation products from cross-linked hydrogel formulations and incubating for 2 or 24 h at 37 °C. Statistically significant differences were determined by a Tukey’s test within time points and a t test across time points (p < 0.05). Leachables from the 10 and 20 wt % TGM hydrogels without PAMAM were found to be cytocompatible under all conditions tested (Figure 9). The leachable products from the PAMAM cross-linked hydrogels demonstrated dose-and time-dependent cytocompatibility at the higher TGM wt % (1×) and lower cross-linker MW (P-1440). The reduced cytocompatibility for the 20 wt % P-1440 group can be attributed to the higher polymer concentration, which increases solution osmolality, and lower MW polymers, which may potentially interact with the cell membrane.

The degradation products from the hydrogel demonstrated short-term cytocompatibility at the higher dilutions but produced significant decreases in cell viability for all dilutions at the later time point as a function of hydrogel polymer content, PAMAM MW, and degradation product dose (Figure 10). For all hydrogel formulations except the 10 wt % P-1440 group, a 1000× dilution group was added because cytotoxicity was observed for the 100× dilution. Exposure to the lower MW products from the PAMAM cross-linker and formation of acidic groups from DBA hydrolysis is one possible explanation for the adverse response. However, the NaOH-catalyzed process to obtain the hydrogel degradation products also may have contributed to the decreased cell viability. The use of strong base to accelerate degradation introduced a significant amount of ions into the system, which could not be easily separated from the actual hydrogel degradation products.

To distinguish the effects between the hydrogel degradation products and the solution osmolality, we performed osmometry on NaCl-treated DMEM and the degradation product dilutions. Figure 11 illustrates the effect of increasing NaCl incorporation on DMEM osmolality and fibroblast viability after 24 h. Increasing the NaCl beyond 0 and 30 mg/10 mL DMEM or an osmolality greater than 450 mOsm led to significant decreases in cell viability after 24 h. In comparison, the 1× dilutions of the 10 wt % P-1440, 10 wt % P-2600, 20 wt % P-1440, and 20 wt % P-2600 groups were extrapolated to be 355, 481, 377, and 539 mOsm, respectively. The 1× values were extrapolated from the other dilutions because the solutions were too concentrated to be measured by the instrument. Several values of solution osmolality observed here fall at the upper limits of the range tolerable by cells (~370–500 mOsm) established in the literature, which has been shown to suppress cell proliferation and induce apoptosis. We postulate that the use of NaOH in the degradation process and administration of all degradation products at once contributed greatly to the negative response in vitro.

Figure 7. Statistically significant interaction effects between DBA mol content (a), PAMAM MW (b), TGM wt % (c), and amine:epoxy mol ratio (d) on the formation (left) and equilibrium (right) weight swelling ratio of hydrogels from the 24 factorial experimental design.

Figure 8. Unconfined compressive Young’s modulus of 6 mol % DBA-containing hydrogels cross-linked with P-1440 PAMAM (6 mm in diameter, 3 mm in height) with varying TGM wt % and amine:epoxy mol ratio. Data are reported as means ± standard deviation for a sample size of n = 6. * and # indicate statistical significance from 10 wt % group at the same amine:epoxy mol ratio and within same TGM wt % at different amine:epoxy mol ratio, respectively.
3.8. MSC Encapsulation. To examine the potential of the hydrogel system for cell delivery in bone tissue engineering applications, we performed a preliminary encapsulation study using rat MSCs at an encapsulation density of 10 million cells/mL for 1 and 7 days. MSCs were able to be successfully encapsulated within the hydrogel using media with serum, which did not negatively affect the cross-linking reactions of the hydrogel. The slight drop in cell viability after the initial week is

Figure 9. Viability of rat fibroblasts in in vitro cytocompatibility testing of TGM and hydrogel leachable products with 6 mol % DBA at 2 and 24 h. The first column corresponds to TGM leachables, and the second and third columns correspond to hydrogel leachables with P-1440 and P-2600 cross-linker, respectively. The top and bottom rows refer to 10 or 20 wt % hydrogels, respectively. Data are reported as means ± standard deviation for a sample size of n = 6. * and # indicate statistical significance (p < 0.05) within and between time points, respectively.

Figure 10. Viability of rat fibroblasts in in vitro cytocompatibility testing of 6 mol % DBA-containing hydrogel degradation products. Rows correspond to the hydrogel TGM wt % (10 or 20 wt %), while columns refer to the PAMAM cross-linker used at a 1:1 amine:epoxy mol ratio (P-1440 or P-2600). Data are reported as means ± standard deviation for a sample size of n = 6. * and # indicate statistical significance (p < 0.05) within and between time points, respectively.
consistent with MSC encapsulation in similar hydrogels in published studies, and the hydrogel was able to support live cells up to 7 days with homogeneous distribution after encapsulation, as shown in Figure 12.

4. CONCLUSIONS

A novel injectable, thermally responsive, chemically crosslinkable, and bioresorbable hydrogel was successfully developed through the synthesis and combination of PNiPAAm-based TGMs with diamine-functionalized PAMAM cross-linkers. The system addresses the main challenges of using PNiPAAm-based hydrogels for tissue engineering, namely, postformation syneresis and minimal resorbability, through the incorporation of a cross-linkable epoxy pendant group and hydrolyzable lactone ring. Hydrogel formation occurred through rapid thermogelation at 37 °C and was stabilized through concomitant amine-epoxy chemical cross-linking over a period of 3 h, creating nonshrinking hydrogels. Additionally, hydrogel degradation was facilitated under accelerated conditions through hydrolysis-dependent processes. Hydrogel swelling was primarily controlled by the overall hydrophilicity and structure of the polymer network, modulated by the content of TGM and PAMAM cross-linker or DBA incorporation, or cross-linker length and density, respectively, while the unconfined compressive modulus was dependent on both the polymer concentration and extent of cross-linking.

The leachable products from the hydrogel system were shown to be cytocompatible for the tested macromer and cross-linked hydrogel concentrations and time scales. Solution osmolality was the primary contributing factor toward the dose- and time-dependent response of the hydrogel degradation products. A preliminary encapsulation study suggested that MSCs could be successfully encapsulated without affecting hydrogel cross-linking and that the hydrogel could support cell viability for up to 7 days. The results suggest that the injectable and thermally and chemically cross-linking hydrogel system studied herein holds great potential for biomedical applications, especially for cell delivery in tissue engineering.

ASSOCIATED CONTENT

Supporting Information

Gelation of TGM and cross-linked TGM 10 wt % hydrogels with P-1440 cross-linker at 1:1 amine:epoxy mol ratio at 37 °C after 1 h and 4 °C in excess PBS pH 7.4 after 3 h. 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


Figure 11. Osmolality of 0, 30, 60, 90, 120, and 220 mg NaCl/10 mL DMEM solutions and cell viability after incubation with the solutions after 24 h. * marks statistical differences in cell viability from the 0 and 30 mg NaCl/10 mL DMEM groups (p < 0.05).

Figure 12. DNA content of acellular and cellular 10 wt % hydrogels with predifferentiated MSCs at an encapsulation density of 10 million cells/mL hydrogel at 1 and 7 days. A representative confocal micrograph of a cellular hydrogel with Live/Dead staining at 7 days. Green and red staining indicate live or dead cells, respectively. Data are reported as means ± standard deviation for a sample size of n = 4. The scale bar represents 100 μm.

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