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Protein-Reactive, Thermoresponsive Copolymers with High Flexibility and Biodegradability

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Abstract

A family of injectable, biodegradable, and thermosensitive copolymers based on *N*-isopropylacrylamide, acrylic acid, *N*-acryloxysuccinimide, and a macromer polylactide–hydroxyethyl methacrylate were synthesized by free radical polymerization. Copolymers were injectable at or below room temperature and formed robust hydrogels at 37 °C. The effects of monomer ratio, polylactide length, and AAc content on the chemical and physical properties of the hydrogel were investigated. Copolymers exhibited lower critical solution temperatures (LCSTs) from 18 to 26 °C. After complete hydrolysis, hydrogels were soluble in phosphate buffered saline at 37 °C with LCSTs above 40.8 °C. Incorporation of type I collagen at varying mass fractions by covalent reaction with the copolymer backbone slightly increased LCSTs. Water content was 32–80% without collagen and increased to 230% with collagen at 37 °C. Hydrogels were highly flexible and relatively strong at 37 °C, with tensile strengths from 0.3 to 1.1 MPa and elongations at break from 344 to 1841% depending on NIPAAm/HEMAPLA ratio, AAc content, and polylactide length. Increasing the collagen content decreased both elongation at break and tensile strength. Hydrogel weight loss at 37 °C was 85–96% over 21 days and varied with polylactide content. Hydrogel weight loss at 37 °C was 85–96% over 21 days and varied with polylactide content. Degradation products were shown to be noncytotoxic. Cell adhesion on the hydrogels was 30% of that for tissue culture polystyrene but increased to statistically approximate this control surface after collagen incorporation. These newly described thermoresponsive copolymers demonstrated attractive properties to serve as cell or pharmaceutical delivery vehicles for a variety of tissue engineering applications.

1. Introduction

Injectable hydrogels as a biomaterial class have been recognized as having a variety of applications in regenerative medicine. They have been extensively explored as vehicles for drug delivery and cell transplantation with the advantage that cells and biomolecules can be readily integrated into the gelling matrix.1 The injectable nature of the hydrogels provides the attractive feature of three-dimensional shape filling prior to gelation, allowing good physical integration, potentially avoiding the need for fixation and facilitating the use of minimally invasive approaches for material delivery. In addition, injectable hydrogels used as biomaterials are primarily aqueous polymer solutions before gelation, avoiding organic

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solvents. Gelation is commonly accomplished by chemical or photochemical cross-linking, although some of these processes may raise cytotoxicity issues or add complexity to delivery systems.[2] Thermoresponsive injectable hydrogels are attractive alternatives because gelation can be realized simply as the temperature increases above the lower critical solution temperature (LCST), which is designed to be below body temperature. Various thermoresponsive and injectable polymers including poly(ethylene oxide)/poly(propylene oxide) and poly(*n*-isopropylacrylamide) (PNIPAAm) copolymers have been developed and employed in a variety of settings.[3–6]

PNIPAAm is a water-soluble polymer possessing an LCST of approximately 32 °C. However, PNIPAAm is nonbiodegrad-able and not readily cleared from the body at normal body temperature, limiting the in vivo applications of such hydrogels. This has provided motivation for the development of biodegradable PNIPAAm-based polymers. Biodegradability can be achieved by incorporating biomacromolecular or peptide sequences into the PNIPAAm-based polymer.[7–10] Ohya et al.[7,8] have developed biodegradable PNIPAAm–gelatin and PNIPAAm–hyaluronic acid. Kim et al.[9] synthesized a collagenase sensitive thermosensitive hydrogel based on *n*-isopropylacrylamide, acrylic acid, and a double-bond-capped peptide Gln-Pro-Gln-Gly-Leu-Ala-Lys. Li et al.[10] conjugated collagen into *N*-hydroxysuccimide containing PNIPAAm copolymer. An alternative approach to introduce biodegradability is to create a copolymer having an LCST below body temperature before degradation and well above body temperature after degradation. This has been achieved by the introduction of hydrophobic oligomers with hydrolytic ester bonds and hydrophilic groups into PNIPAAm copolymers. The labile hydrophobic groups temporarily decrease the LCST and the hydrophilic groups increase the LCST above body temperature after hydrolysis.[11,12]

Our objective in this work was to synthesize a thermoresponsive hydrogels having robust mechanical properties that would be appropriate for a variety of soft tissue cell therapy applications such as myocardium, muscle, and blood vessel. Specifically, we desired a material that would be readily injectable (soluble) at low temperatures, would form a gel with relatively high tensile strength and distensibility at 37 °C, would hydrolyze to form soluble, nontoxic degradation products, and would be capable of reacting with biomolecules such as collagen to impart bioactivity. To accomplish these objectives a family of thermoresponsive copolymers was synthesized based on NIPAAm, polylactide–hydroxyethyl methacrylate (HEMAPLA), acrylic acid (AAc) ,and *N*-acryloxysuccinimide (NAS). The macromer HEMAPLA was used to introduce biodegradability by increasing backbone solubility upon PLA hydrolysis. AAc was employed to manipulate polymer hydrophilicity and NAS provided bioconjugation sites for biomolecular binding. Type I collagen was conjugated into the resulting hydrogels at varying concentrations to putatively improve cytocompatibility. The chemical, mechanical, and cytocompatibility properties of the resulting copolymers were assessed.

2. Experimental Methods

2.1. Materials

Chemicals were purchased from Sigma-Aldrich unless otherwise stated. $_{D,L}$-lactide and NIPAAm were purified by recrystallization in ethyl acetate and hexane respectively and vaccuum-dried before use. AAc was distilled under reduced pressure. *N*-acryloxysuccinimide (NAS), benzoyl peroxide (BPO), and stannous 2-ethylhexanoate [$Sn(OCt)_2$] were used as received. 2-Hydroxyethyl methacrylate (HEMA, Sigma) was used after processing with an inhibitor removal column (Sigma). Acid-soluble type I collagen was purchased from MP Biomedicals and dissolved in acetic acid solution to obtain a 4 wt % solution. Solvents used in the experiments were analytical grade and were used as received.

2.2. Synthesis of HEMA-Polylactide (HEMAPLA) Macromer

Macromer HEMAPLA was synthesized by ring-opening polymerization of lactide with 2-hydroxyethyl methacrylate with stannous octoate as catalyst (Scheme 1).[13] Stoichiometric amounts of lactide and HEMA were mixed in a 250 mL three-neck flask. Stannous octoate (1 mol % with respect to HEMA) in 1 mL of toluene was added subsequently. The reaction was conducted at 110 °C for 1.5 h under a nitrogen atmosphere. The mixture was then dissolved in THF and precipitated in cool water. The precipitate was collected by centrifugation, dissolved in ethyl acetate, and dried with $MgSO_4$ overnight. Ethyl acetate was evaporated under reduced pressure. The yielded viscous oil was dried in a vacuum oven overnight. The feed ratio of HEMA to lactide was varied from 1/1, 1/2, and 1/3 to obtain macromers with different PLA lengths.

2.3. Synthesis of poly(NIPAAm-*co*-AAc-*co*-NAS-*co*-HEMAPLA)

Poly(NIPAAm-*co*-AAc-*co*-NAS-*co*-HEMAPLA) copolymers were synthesized by free radical polymerization (Scheme 2) in a 250 mL one-necked round-bottom flask. Stoichiometric amounts of monomers (NIPAAm, AAc, NAS, and HEMAPLA) were dissolved in 1,4-dioxane to form a 10 wt % monomer solution. The solution was bubbled with an argon atmosphere for 10 min. The flask was then sealed. Degassed initiator benzyl peroxide (BPO, 7.2×10^{-3} mol/mol monomer) in 1,4-dioxane was subsequently injected into flask. The polymerization was carried out at 70 °C for 24 h. The polymer solution was cooled to room temperature, precipitated in hexane, filtered, and then dried in a vacuum oven overnight. The copolymer was purified twice by precipitation from THF into diethyl ether. The purified copolymer was vacuum-dried at 50 °C for 2 days.

2.4. Bioconjugation of Collagen with Copolymers

Bioconjugation between the copolymer and type I collagen was conducted at 4 °C (Scheme 3). A defined amount of collagen solution (4 wt % in acetic acid) was neutralized with small aliquots of 2.0 M sodium hydroxide solution. The neutralized collagen was thoroughly mixed with 20 wt % copolymer in phosphate buffered saline (PBS, pH = 7.4) in an ice/water bath. The mixture was then transferred into a 4 °C refrigerator, and conjugation was carried out for 24 h. The collagen content initially added in the hydrogel was varied from 5 to 20 wt %. To confirm the reaction of NHS groups in the polymer, hydrogels before and after collagen incorporation were characterized with ATR-FTIR. Hydrogels with or without collagen were set in a 4 °C refrigerator for 24 h and were then allowed to gel at 37 °C followed by washing three times with PBS. The hydrogels were then placed in a Nicolet FTIR spectrometer equipped with ATR and the spectra were recorded.

2.5. Polymer Characterization

Polymer molecular weight was determined by gel permeation chromatography (GPC, Waters Breeze system, Waters 1515 HPLC pump, Waters 2414 differential refractometer), using monodisperse poly(methylmethacrylate) standards for calibration. Measurements were made at 50 °C with dimethyl formide as a solvent. The injection volume was 200 μL, and the polymer concentration was approximately 1 mg/mL. Fourier transform infrared (FTIR) spectra were obtained at room temperature with a Nicolet FTIR spectrometer. A 5% solution of polymer in chloroform was placed directly onto the NaCl window with subsequent evaporation of chloroform at 50 °C. ^{1}H NMR spectra were recorded with a 300 MHz spectrometer using DMSO-d_6 as a solvent. Copolymer LCSTs were determined by differential scanning calorimetry (DSC-60; Shimadzu) with a scanning rate of 10 °C/min over a range of 0–80 °C. The temperature at the maxima of the endotherm peak was taken as the LCST.[14] Copolymer solutions and copolymer/collagen bioconjugates were tested. Copolymers solutions were formed by dissolving copolymers in PBS (pH = 7.4) at 20 wt %.

LCSTs of completely hydrolyzed copolymers were measured after hydrolysis in a 1.0 M sodium hydroxide solution at 4 °C for 10 days, followed by neutralization with a 10 M hydrochloride solution. The molecular weight of one of the polymers (NIPAAm/AAc/NAS/HEMAPLA ratio of 80/11/5/4 with 3.9 lactide units) was also measured after the same hydrolysis period.

The water content was measured for hydrogels formed at 37 °C. A 20 wt % copolymer solution in PBS (pH = 7.4) or copolymer/collagen bioconjugate was placed in a 37 °C water bath to allow gelation. The hydrogel was maintained at 37 °C for 5 h and was then removed and wiped with tissue paper to remove the surface water. Water content was defined as the normalized difference of the wet mass (w_2) and dry mass of the hydrogel (w_1).

$$\text{water content } (\%) = 100 \times (w_2 - w_1)/w_1$$

Four independent measurements were performed.

To measure hydrogel tensile properties, 1 mL of 20 wt % of copolymer solution or copolymer/collagen conjugate solution was first placed in a 20 mL scintillation vial and then in a 37 °C water bath for 5 h. Hydrogel samples of uniform thickness were cut into a rectangular shape with a width of 4 mm and a length of 25 mm. The samples were loaded by gripping both ends into the test machine, whose grips were preheated in a 37 ± 2 °C water bath. The length between the two grips was greater than 18 mm. The testing was conducted with an ATS 1101 universal testing machine equipped with a 10 lb load cell. A crosshead speed of 10 mm/min was used. Five samples were evaluated for each polymer composition.

2.6. Hydrogel Degradation

Hydrogel degradation was quantified by mass loss under aqueous conditions. Hydrogels were cut at 37 °C and quickly frozen at −80 °C, after which they were lyophilized and then vaccuum-dried at 50 °C. Dry samples (~60 mg) were weighed (w_1) and warmed at 37 °C, then immersed in a scintillation vial containing 5 mL of 37 °C PBS (pH = 7.4). The degradation was conducted at 37 °C in a water bath. Samples were taken at intervals, rinsed with 37 °C water, dried in a vacuum oven for 2 days at 50 °C, and weighed (w_2). The weight remaining was calculated as:

$$\text{water remaining } (\%) = 100 \times w_2/w_1$$

2.7. Cytotoxicity Assay of Degradation Products

The cytotoxicity of copolymer degradation products was assessed as described previously.[15,16] Rat vascular smooth muscle cells (RSMCs) were isolated according to the method of Ray et al. from animals that had been utilized in other protocols.[17] Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) to the fifth passage and seeded into a 96-well tissue culture plate at 1.5×10^5/mL. The culture medium (200 μL) and completely degraded copolymer solution (33 μL) were mixed to obtain a degradation product concentration of 1.7 mg/mL. For control experiments, culture medium alone was used. After 48 and 96 h of RSMC culture, cell viability was measured ($n = 4$) using a colorimetric tetrazolium salt (MTT) assay for mitochondrial activity.

2.8. Smooth Muscle Cell Adhesion on Hydrogels

Rat smooth muscle cells were seeded onto hydrogel surfaces to evaluate their ability to support cell growth. Cells at the fifth passage were used. Hydrogels with or without collagen were cut into 6 mm diameter disks and placed into 96-well tissue culture plate wells. RSMCs were seeded at a density of 3×10^5/mL and cultured for 24 h. Cell viability was evaluated with the mitochondrial assay ($n = 4$), with tissue culture polystyrene surfaces employed as a control.

2.9. Smooth Muscle Cell Encapsulation in Hydrogels

To evaluate cell viability after encapsulation within the hydrogel matrices, smooth muscle cells were first labeled with living cell marker CellTracker Green CMFDA (5-chloromethylfluorescein diacetate, Invitrogen) by exposing a cell suspension to culture medium to which CMFDA in DMSO was added at a final concentration of 10 μM. The cell suspension was incubated at 37 °C for 15 min, followed by centrifugation. The cell pellet was resuspended in culture medium and washed twice to remove free CMFDA. A 20% p(NIPAAm-*co*-AAc-*co*-NHS-*co*-HEMAPLA3.9) PBS solution with or without 5% collagen was precooled to 4 °C and exposed to UV irradiation in the laminar flow hood for 30 min. A labeled cell suspension (0.25 mL of 2×10^7 cells/mL PBS) was then mixed thoroughly with 1 mL of the polymer solution. The mixture was transferred into a 37 °C water bath for gelation for 10 min. The water expelled from the hydrogel was removed and replaced with an equal volume of medium comprising PBS and 20% FBS. The medium was changed daily for 7 days. The hydrogel was cut into ~100 μm thick pieces and visualized with fluorescence microscopy. The mechanical properties of the RSMC encapsulated hydrogels were tested 10 min after gelation according to the method described in Section 2.4. Five samples were evaluated for each hydrogel.

2.10. Statistical Methods

Data are expressed as mean ± standard deviation. Statistical analysis was performed by ANOVA with post hoc Neuman–Keuls testing for differences. For hydrogel degradation data, hydrogels with different lactate length and PNIPAAm/HEMAPLA ratio were compared with repeated measures by ANOVA to evaluate the effect on weight loss.

Results

3.1. Synthesis of Macromer and Copolymer

The synthesis of HEMAPLA macromer was confirmed by ^{1}H NMR (Figure 1) with evidence of proton peaks from the HEMA residue (a, b, c, d, and e) and lactyl residues (f, g, h, and i). The number of lactate units in HEMAPLA was calculated from the ratio of the integrals of methine in lactate at 5.2 ppm and the double bond in HEMA at 5.7–6.0 ppm. The numbers of lactate units in the HEMAPLA macromers were 2.1, 3.9, and 7.0, corresponding to feed ratios of 1/1, 1/2, and 1/3, respectively.

Copolymers with different monomer ratios were prepared by free radical polymerization (Scheme 2). FTIR spectra of the copolymers (not shown) exhibited broad and weak carboxylic group absorption at 1713 cm^{-1}, ester group absorption at 1730 cm^{-1}, and succinimide group absorption at 1763 cm^{-1} together with weak peaks at 1795 and 1812 cm^{-1}. Figure 2 shows the typical ^{1}H NMR spectrum from one copolymer. Characteristic proton peaks are seen from each monomer, NIPAAm (c, d, e, d, f, and k), AAc (e and f), NAS (a, e, and f), and HEMAPLA (b, e, h, i, and g). Copolymer compositions were calculated based on the integration ratio of characteristic proton peaks from each monomer, NIPAAm (c), NAS (a/2), AAc [$f - (c + a/4)$], and HEMAPLA (b/2). Monomer feed ratios and copolymer compositions are listed in Table 1. The alteration of the number of lactide

units, HEMAPLA content, and AAc content by altering the feed ratio would be used to examine the effects of these variables on the indices measured below. The compositions determined by ^{1}H NMR were found to be consistent with the feed ratios.

3.2. LCST

All the synthesized copolymers were soluble in PBS at 4 °C, and 20 wt % polymer solutions could easily be injected through an 18 gauge needle at 4 °C. For a smaller 22 gauge needle, all of the solutions except the ones with NIPAAm/ AAc/NHS/HEMAPLA ratios 75/6/5/14 could also be easily injected. LCSTs determined from DSC curves for each copolymer are presented in Table 2. All of the copolymers possessed LCSTs well below 37 °C, ranging from 26.1 to 18.4 °C depending on copolymer composition. When placed in a 37 °C water bath, polymer solutions formed hydrogels within 5 s. Comparing copolymers with the same PLA and AAc content, the LCST decreased with a decrease in the NIPAAm/HEMAPLA ratio. An increase in the PLA length was associated with a decrease in the LCST.

Copolymers were hydrolyzed with NaOH to remove the degradable lactate residue, theoretically mimicking the polymer after complete degradation in PBS. The hydrolyzed copolymers demonstrated an increase in LCSTs to well above 37 °C in all cases. Copolymers with the same monomer ratios showed similar LCSTs after hydrolysis. The change of NIPAAm/ HEMAPLA ratio did not change the LCST remarkably, whereas an increase of the AAc content from 6% to 11% largely increased LCST from 41 °C to above 60 °C.

3.3. Bioconjugation of Collagen with Copolymer

The conjugation of collagen with hydrogel was indirectly confirmed by ATR-FTIR (Figure 3). Hydrogels without collagen showed characteristic NHS group peaks at 1740 and 1780 $cm^{-1,}$ respectively (Figure 3, curve A). Hydrogels incorporating collagen did not exhibit these two peaks (Figure 3, curve B), implying that collagen participated in a reaction with the NHS groups. The bioconjugation of collagen to the various copolymers variably affected the ability of the resulting composites to form hydrogels (Table 3). When the collagen content was 20%, none of the composites were able to form hydrogels but rather precipitated to form a suspension. Mixing 5 or 10% collagen with the copolymers resulted in hydrogel formation in all cases examined. These composites were still injectable at low temperature, for instance 4 °C. Collagen incorporation slightly increased the LCST as compared to the respective unconjugated copolymer. For example, the copolymer with a feed ratio of 80/6/5/9 had an LCST 21.0 °C and the addition of 5 and 10% collagen increased the LCST to 21.2 and 22.4 °C respectively.

3.4. Water Content

The water content of hydrogels incubated for 5 h at 37 °C in PBS is presented in Figure 4. Looking at a later time point of 12 h, no significant differences were found from the 5 h data. The water content was found to be related to the NIPAAm/HEMAPLA ratio, PLA length, AAc content, and collagen content. In Figure 4A, for copolymers with the same feed ratio, an increase in lactate units from 2.1 to 3.9 significantly decreased the water content ($p < 0.05$). Comparing copolymers with the same lactate length, the decrease of NIPAAm/ HEMAPLA from 85/4 to 75/14 also significantly decreased water content ($p < 0.05$). In Figure 4B, it is seen that an increase in the AAc content from 6% to 11% significantly increased the water content ($p < 0.05$). The conjugation of collagen into the copolymer markedly increased water absorption ($p < 0.05$). With the increase of collagen content from 0% to 5% to 10%, the water content increased from 38% to 163% to 231% (Figure 4C).

3.5. Mechanical Properties

Hydrogel mechanical properties at 37 °C under aqueous conditions are presented in Figure 5 and Figure 6. Figure 5 shows the typical stress–strain curves for one of the hydrogels with or without collagen. Qualitatively, the hydrogels were found to be highly flexible and relatively strong. Hydrogels without collagen possessed tensile strengths greater than 0.5 MPa and elongations at break exceeding 1330%. Hydrogel mechanical properties were dependent upon the NIPAAm/HEMAPLA ratio, AAc content, and lactate length. In Figure 6A, it is seen that decreasing the NIPAAm/HEMAPLA ratio from 85/4 to 80/9 significantly improved tensile strength ($p < 0.05$) while further decreasing the ratio to 75/14 decreased tensile strength ($p < 0.05$). However, decreasing the NIPAAm/HEMAPLA ratio increased the elongation at break from 1398% to 1842% ($p < 0.05$ for 85/4 versus 75/14). The modulus also increased with a decrease in NIPAAm/HEMAPLA ratio ($p < 0.05$). The tensile strength was decreased from 0.60 ± 0.10 to 0.55 ± 0.06 MPa when the AAc content was increased from 6% to 11% in copolymers with 85/6/5/4 (2.1) and 85/11/ 5/4 (2.1) monomer ratios, respectively ($p > 0.05$), whereas elongation at break increased from 1398 ± 87 to 1580 ± 138% ($p < 0.04$). An increase in AAc content insignificantly decreased the hydrogel modulus from 53 ± 12 to 46 ± 6 kPa ($p > 0.05$). At the same NIPAAm/HEMAPLA ratio of 85/6/5/4, increasing the number of lactate units from 2.1 to 3.9 increased the tensile strength from 0.6 ± 0.1 to 1.1 ± 0.04 MPa ($p < 0.01$) while elongation at break decreased from 1398 ± 87 to 1196 ± 63% ($p < 0.05$) and the modulus increased from 53 ± 12 to 117 ± 24 kPa ($p < 0.05$).

Collagen addition reduced hydrogel mechanical properties (Figure 6B). Both tensile strength and elongation at break decreased with the increase of collagen content ($p < 0.05$). However, elongation at break and tensile strength remained greater than 344% and 0.3 MPa, respectively. The addition of 5% and 10% collagen in the hydrogel did not significantly change modulus ($p > 0.05$ for 0 versus 5%, 0, and 10% and 5% versus 10%).

Figure 7 shows macroscopic images of the morphological changes in one of the hydrogels [85/6/5/4 (2.1)] after stretching and temperature change in an aqueous environment. In Figure 7A, a strain of 100% was applied, followed by a recovery period where 91% strain recovery was observed. When the stretched hydrogel was cooled from 37 to 22 °C, the material fractured. Further cooling to 4 °C resulted in hydrogel solubilization in water (Figure 7B).

3.6. Hydrogel Degradation

All of the studied hydrogels showed significant mass loss exceeding 85% when incubated in PBS at 37 °C over 21 days. Figure 8 shows the degradation behavior of hydrogels with various NIPAAm/HEMAPLA ratios and PLA lengths. For hydrogels with PLA lengths of 2.1, the decrease of NIPAAm/HEMAPLA ratio did not significantly affect weight loss during a 21 day degradation period ($p > 0.05$). However, when the PLA length was 3.9, a decrease in the NIPAAm/HEMAPLA ratio significantly decreased the mass loss during the degradation period ($p < 0.05$). With the same NIPAAm/HEMAPLA ratio, an increase in the PLA length from 2.1 to 3.9 decreased mass loss in the first 7 days ($p < 0.05$). The molecular weight of one of the completely hydrolyzed polymers (NIPAAm/AAc/NAS/HEMAPLA ratio of 80/11/5/4 with 3.9 lactide units) was measured and found to decrease relative to the nondegraded polymer with M_n before hydrolysis at 35919 and after hydrolysis at 29828. In Figure 9 are FTIR spectra of original polymer and partially degraded polymer without neutralization with hydrochloride. The original polymer showed characteristic peaks of amide, carboxyl, and ester groups at 1650, 1735, and 1758 $cm^{-1,}$ respectively. The partially degraded polymer also showed characteristic peak of the ester group at 1758 cm^{-1}. However, two peaks at 1650 and 1685 cm^{-1}, respectively, were seen for amide groups, and

the peak at 1720 cm^{-1} was seen for the carboxyl group, probably due to chemical environment change for the partially degraded polymer.[18] When compare the absorption ratio of the ester group and the amide group, it was found that the original polymer had a higher ratio than that of degraded polymer, confirming that the ester groups were partially degraded.

3.7. Cytotoxicity of Degradation Products

Smooth muscle cell viability under culture media with or without supplementation with copolymer degradation products allowed initial evaluation of degradation product cytotoxicity. With the un-supplemented medium resulting in a cell density defined as 100% at each time point, the degradation product-containing medium resulted in cell densities of 109 ± 5 and 99 ± 9% after 2 and 4 days of culture, respectively. The cell densities were not significantly different between control and degradation product-containing medium ($p > 0.05$) at both time points.

3.8. Hydrogel Cytocompatibility

Two-dimensional smooth muscle cell culture on the hydrogel surface was performed to evaluate its ability to support cell adhesion. The adhered and viable smooth muscle cells 24 h after cell seeding onto hydrogel surfaces with or without collagen was quantified by MTT assay. Considering the adherent cell density onto a tissue culture polystyrene plate to be 100%, the adhesion on the hydrogel without collagen was about 29% ($p < 0.01$). For the hydrogel with 5% collagen, the 24 h cell viability adhesion was improved to 97% of that on tissue culture polystyrene ($p < 0.01$ versus 0% collagen).

Three-dimensional smooth muscle cell culture was conducted by encapsulation of cells within the hydrogel to evaluate its ability to support cell survival and growth. Hydrogels containing 0 and 5% collagen had encapsulation efficiencies of 92 ± 4% and 95 ± 2%, respectively. Cell encapsulated hydrogels without collagen had a tensile strength 0.66 ± 0.10 MPa and elongation at break of 1600 ± 135%, while cell encapsulated hydrogels containing 5% collagen exhibited a tensile strength of 0.44 ± 0.04 MPa and elongation at break of 850 ± 81%. Cell density was high for both hydrogels after encapsulation (Figure 10A,B). Cells remained viable during the 7 day culture period and cell density did not change obviously after 3 days (Figure 10C,D and Figure 11). After 7 days, the collagen containing hydrogel exhibited higher cell density than non-collagen-containing hydrogel (Figure 10E,F and Figure 11).

Discussion

Our objective was to synthesize a thermoresponsive hydrogel that would be readily injectable at low temperatures, would form a gel with relatively high tensile strength and distensibility at 37 °C, would hydrolyze to form soluble, nontoxic degradation products, and would be capable of reacting with biomolecules such as collagen to impart bioactivity. This was accomplished through the molecular design of a copolymer where component ratios or the characteristics of one component (HEMAPLA) were manipulated to achieve the desired functionality.

Copolymer Design Effects on the LCST

Copolymer LCSTs before and after degradation were manipulated by polymer hydrophilicity/hydrophobicity ratios. It was necessary to increase the PNIPAAm LCST from 32 °C to above 37 °C so that the polymer would be soluble following cleavage of the polylactide residues on the HEMAPLA. AAc, a hydrophilic component that has been shown to raise the LCST,[12,19] was successfully employed to accomplish this objective. Earlier

reports have shown that introduction of hydrophobic components can decrease the LCST of PNIPAAm.[20] Introduction of the degradable hydrophobic polylactide segment was able to decrease the LCST below 37 °C so that gelation would occur at body temperature prior to hydrolysis. Before copolymer degradation, the LCST was sensitive to the PLA length and HEMAPLA content. With increases in the PLA length and relative HEMAPLA content, the LCST decreased. This was attributed to an increase in copolymer hydrophobicity that facilitated the dehydration of polymer chains. After incubation with NaOH, the PLA oligomer in the HEMAPLA residues should be completely degraded, leaving the hydrophilic polyHEMA structure. Table 2 shows that copolymers with the same composition but different PLA lengths prior to NaOH hydrolysis had similar LCSTs after degradation. In addition, LCSTs did not show a marked dependence on HEMAPLA content after degradation. This is consistent with previous reports that HEMA content did not affect the LCST of poly(NIPAAm-*co*-HEMA).[20] When copolymers had the same NIPAAm/HEMAPLA ratio, increasing the AAc content appeared to slightly increase the LCST before degradation; however, LCSTs were remarkably increased after degradation. This could be attributed to the hydrophobicity effect on the LCST being dominant before degradation, whereas after degradation, AAc played a major role in increasing the LCST because it would act to inhibit dehydration of the polymer chains. It has previously been shown that increasing the AAc content of PNIPAAM copolymers can linearly increase the LCST.[14]

Collagen Incorporation

The ability to covalently conjugate biomolecules into the hydrogel was accomplished by the inclusion of amine reactive NAS residues in the copolymer and demonstrated by coupling type I collagen into the hydrogels, putatively through free amines on collagen lysyl residues. As one might expect, collagen content affected hydrogel properties. ATR-FTIR spectrum in Figure 3 indirectly confirmed that collagen was conjugated in the hydrogel. It is also possible that there may also have been physically entangled collagen present. Because our goal was to use collagen to improve cytocompatibility, the physically entangled collagen, if present, would not compromise the cytocompatibility improvement. Conjugation of 5 and 10% collagen allowed effective formation of hydrogels. However, when the collagen fraction was increased to 20%, all of the copolymer/collagen composites failed to form hydrogels and instead precipitated to form emulsions. This latter result was attributed to interactions between collagen and copolymer molecules acting to interrupt self-associative copolymer chain interactions, thus preventing effective chain entanglement and gel formation. The increase observed in the LCSTs after collagen conjugation was attributed to increased overall hydrogel hydrophilicity.

Hydrogel Water Absorption Characteristics

The synthesized copolymers possessed hydrogel characteristics at 37 °C with water contents greater than 30%.[21] As one would expect, the water content was related to the HEMAPLA content and PLA length. When the PLA length and HEMAPLA content were increased, hydrogel hydrophobicity increased, repelling more water from the hydrogel and resulting in decreased water contents. Others have reported that, for PNIPAAm-based hydrogels, water content is related to temperature even beyond the LCST, with water content decreasing as the temperature increases.[22,23] This temperature effect would generally lead to an expectation that hydrogels possessing a lower LCST would be in a more hydrophobic state and have lower water content at a given temperature above the LCST. Figure 4 showed that increasing the copolymer AAc content increased hydrogel water content, presumably due to an increased repulsion between carboxylate groups. In addition, hydrogels with higher AAc content showed higher LCSTs, which decreased the temperature difference as compared to low AAc content hydrogels, therefore the water loss was reduced at temperatures beyond the

LCST. After collagen conjugation, copolymers were also shown to have significantly higher water content, attributable directly to collagen hydrophilicity.

Mechanical Properties

Hydrogels exhibited attractive mechanical properties with elongations at break higher than 1330% and tensile strengths greater than 0.6 MPa before collagen conjugation and elongations at break higher 350% and tensile strengths greater than 0.3 MPa after collagen conjugation. Strain recovery and high flexibility was qualitatively observed for all of the hydrogels examined. The measured tensile properties are greater than those reported for collagen gels[24] and polyvinyl alcohol (PVA) based hydrogels.[25] The mechanical properties were mainly affected by HEMAPLA content and PLA length. Increases in these parameters decreased hydrophilicity and water content in the hydrogel, which would act to increase copolymer intermolecular interactions and increase tensile strength. Increasing the AAc content or incorporating collagen into the hydrogel increased the water content and thus decreased copolymer chain interactions, leading to decreased tensile strength.

Hydrogel Degradation and Cytocompatibility

All of the hydrogels experienced relatively fast degradation and solubilization with mass losses ranging from 85% to 95% over 21 days. Hydrogels with higher PLA content experienced slower mass loss, attributable to their decreased water content and thus decreased access of hydrolytically labile ester bonds to water. This degradation might be slowed by adding length to the attached PLA segments or increasing HEMAPLA content, evidence for which was observed in Figure 8. Decreasing the AAc content might also have a slowing effect on mass loss, however this was not examined. Copolymer design alterations aimed at slowing the degradation rate would be concomitant with impacts on other copolymer properties such as the LCST and tensile strength. The cell encapsulating hydrogel may experience faster degradation rates during longer term culture because cell secreted enzymes may degrade collagen conjugated in the hydrogel as well as the hydrogel itself. Countering this effect could be the elaboration of extracellular matrix during culture.

Completely solubilized copolymer demonstrated a lack of measurable cytotoxicity on smooth muscle cells in culture. Cell adhesion studies showed that the hydrogel alone did not support extensive cell attachment, an expected result due to its high hydrophilicity. Conjugation of collagen into the hydrogel to provide adhesive ligands remarkably improved cell adhesion levels, raising them to approximate that of tissue culture polystyrene. The hydrogels were also shown to exhibit high cell encapsulation efficiencies, supporting cell viability over a one week culture period. These results suggest that the materials appear to be promising to investigate as cell carriers in regenerative medicine approaches, for instance as vehicles to deliver smooth muscle cells for myocardium cell therapy[26] and chondrocyte for cartilage regeneration,[27] although evaluation of specific cell types for cytocompatibility evaluation would be necessary.

Earlier Reports and Study Limitations

The copolymers described here build upon several previous reports in the field of thermoresponsive polymers. Specifically, Neradovic et al. reported the synthesis of a thermoresponsive hydrogel poly(NIPAAm-*co*-HEMAPLA) that would incorporate hydrolytically labile branches into the copolymer. The final degradation product in this report had an LCST similar to that of PNIPAAm.20 With an LCST lower than 37 °C, the hydrolyzed polymer would presumably not be solubilized after degradation in vivo. Vernon et al. developed a thermoresponsive hydrogel similar to that of Neradovic, but incorporating and manipulating AAc in their copolymer.[11] These hydrogels showed a time (degradation) dependent LCST change with LCSTs higher than 37 °C after degradation. However,

hydrogels based on NIPAAm, HEMAPLA, and AAc alone showed very quick degradation and solubilization rates, with most solubilizing within three days.[12] Tensile mechanical properties were not reported for these materials. Structurally, the major difference between the current work and these previous reports is the incorporation of bioconjugation abilities through the introduction of NAS into the copolymer and the subsequent investigation of this new family of copolymers with and without collagen conjugation.

The current work has several limitations worth citing. First, the developed hydrogels still possess relatively fast degradation rates, even though these have been substantially extended with respect to previous studies.11,[12] Although there would be applications where a relatively fast degradation rate would be desired, for applications requiring slower dissolution, a slower degrading polyester-based macromer might be evaluated because the hydrogel degradation rate is controlled by the degradation of the polyester segment in the macromer. Second, we have only examined the conjugation of type I collagen here, but future studies with other biomacromolecules (e.g., growth factors, differentiation factors, adhesive proteins) would be of interest, particularly in concert with cell encapsulation. The controlled release properties of the conjugated hydrogel system for pharmaceutical delivery would merit study. Finally, we have not examined the behavior of these hydrogels in vivo. One would expect that hydrolysis and solubilization rates would differ in vivo, and this could be a function of local transport at the site of implantation. Also, although cytotoxicity does not appear to be of concern, toxicity in vivo remains to be evaluated. Previous reports with PNIPAAM derivatives would suggest that this may not be a major concern.[10,28]

In summary, we have synthesized a family of injectable and thermoresponsive copolymers that are capable of conjugation to type I collagen and are mechanically robust. Copolymer design parameters were shown to affect the resulting hydrogel LCSTs, water absorption ratios, and mechanical properties in a generally predictive manner. These copolymers might find future application in the encapsulation and delivery of cells as well as for the coupling of bioactive molecules through the amine-reactive functionality of the polymer backbone for subsequent in situ delivery.

Acknowledgments

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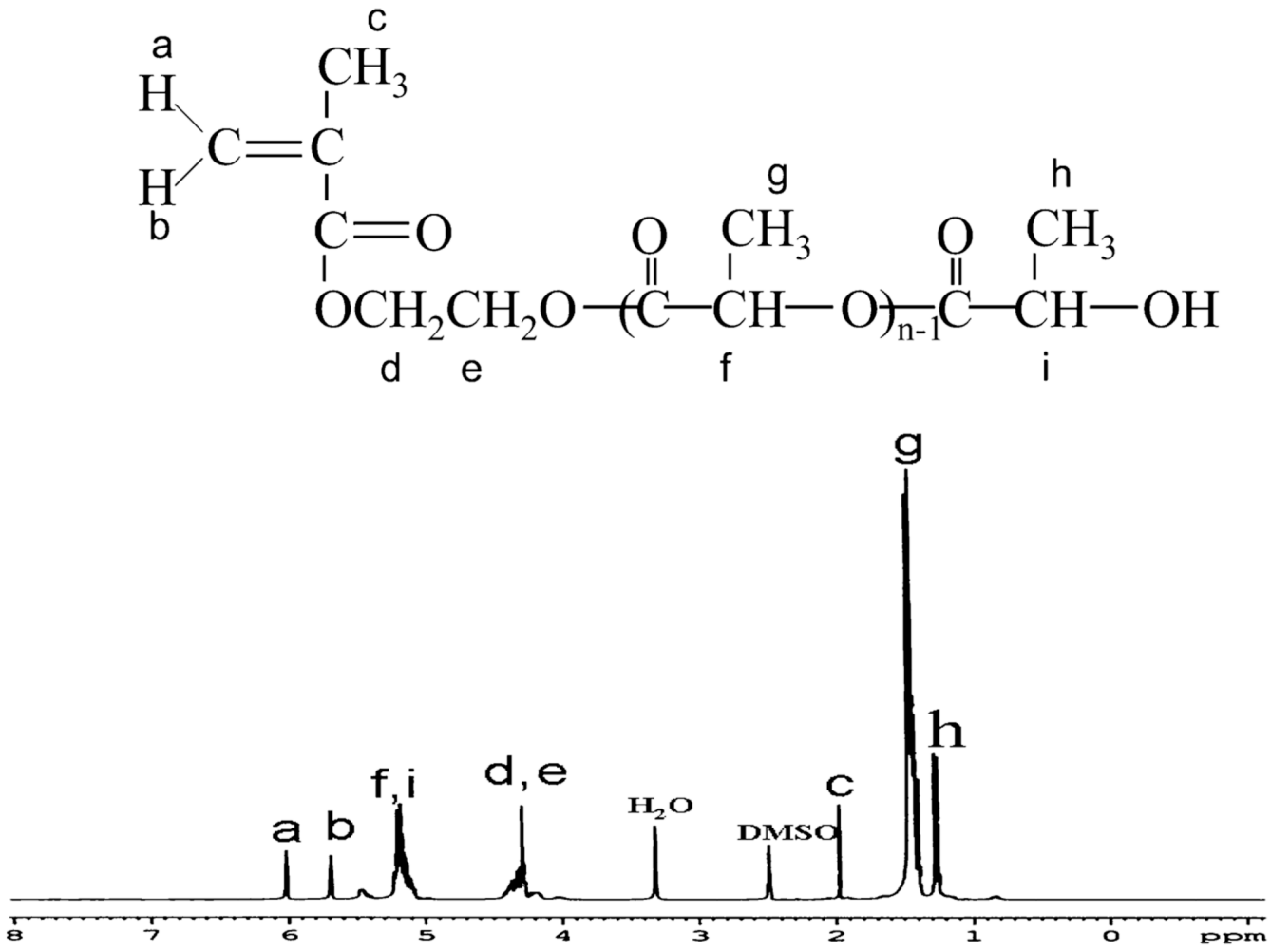


Figure 1.
^{1}H NMR spectrum of HEMAPLA macromer.

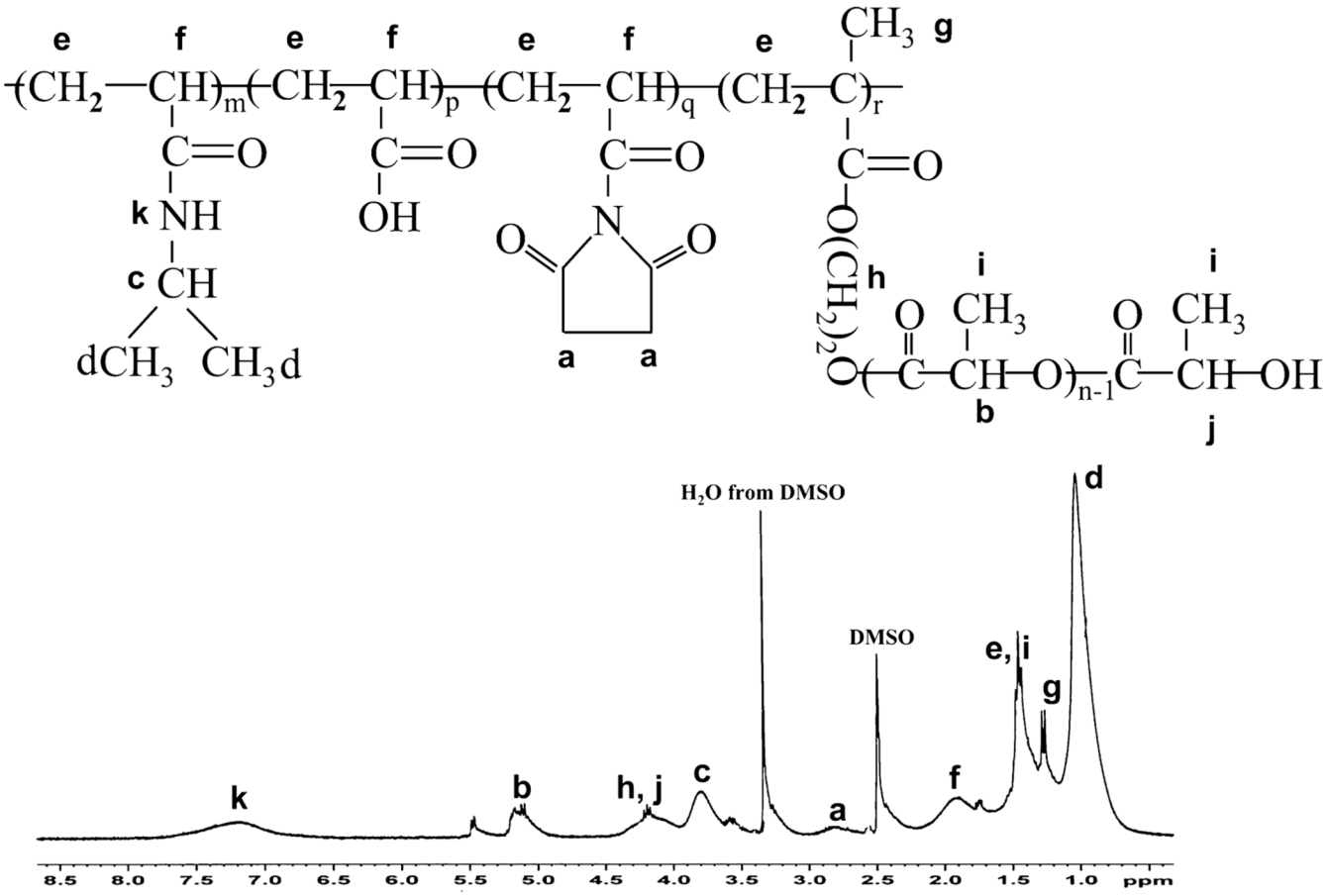


Figure 2.
^{1}H NMR spectrum of poly(NIPAAm-*co*-AAc-*co*-NAS-*co*-HEMAPLA).

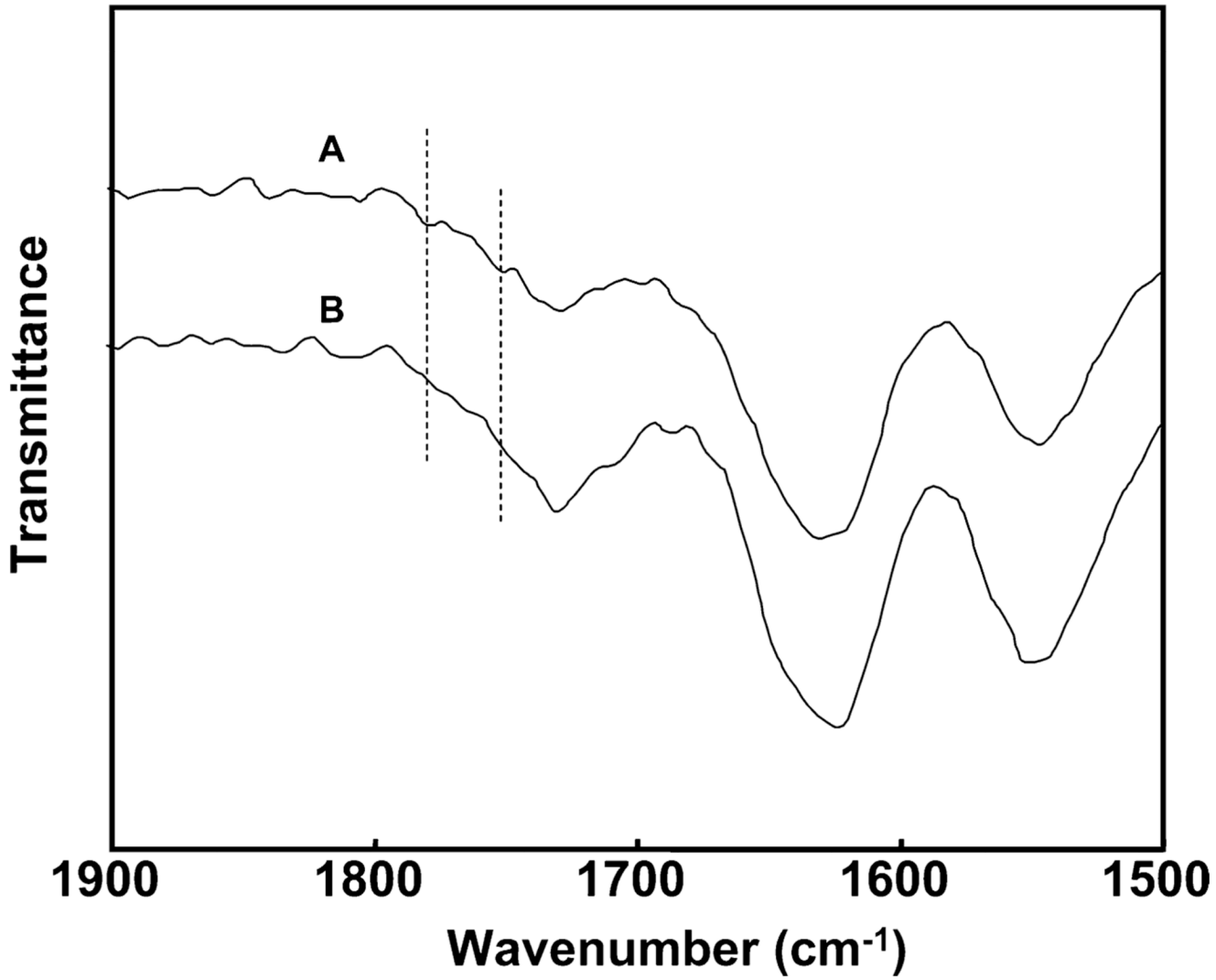


Figure 3.
ATR-FTIR spectra of poly(NIPAAm-*co*-AAc-*co*-NAS-*co*-HEMAPLA) before (A) and after (B) collagen conjugation. Hydrogels without collagen showed characteristic NHS group peaks at 1740, 1780 cm^{-1}, respectively, which were not present after collagen conjugation.

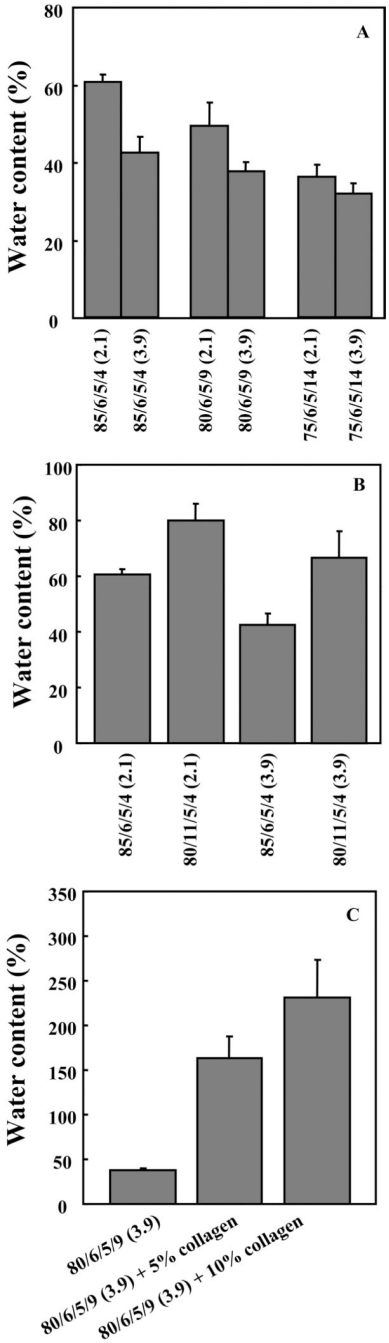


Figure 4.

Effect of polymer composition on water content. (A) effect of PLA length and NIPAAm/HEMAPLA ratio, (B) effect of AAc content, (C) effect of collagen content.

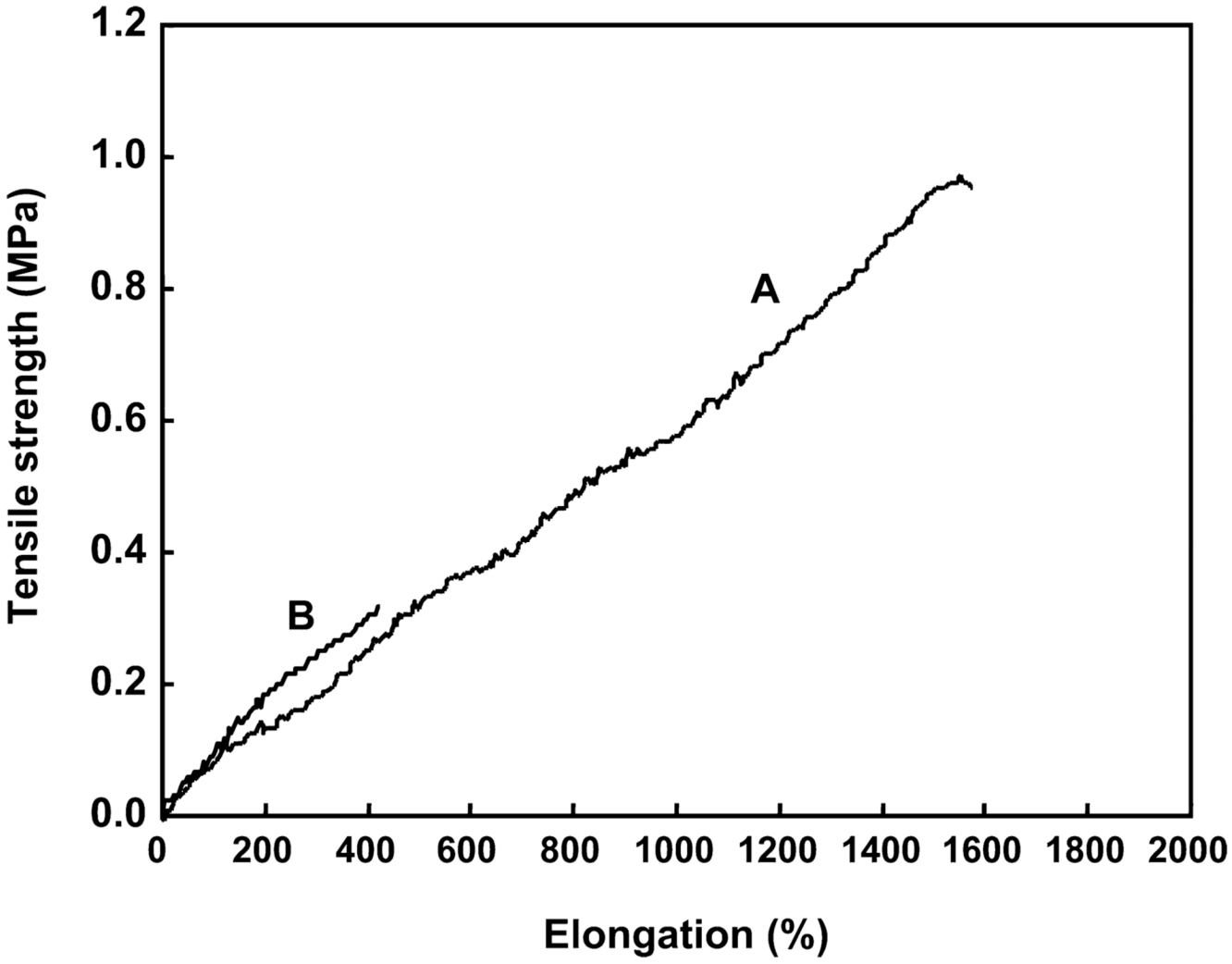


Figure 5.
Typical stress–strain curves for poly(NIPAAm-*co*-AAc-*co*-NAS-*co*-HEMAPLA) (80/6/5/9 (2.1)) without (A) and with (B) 5% collagen.

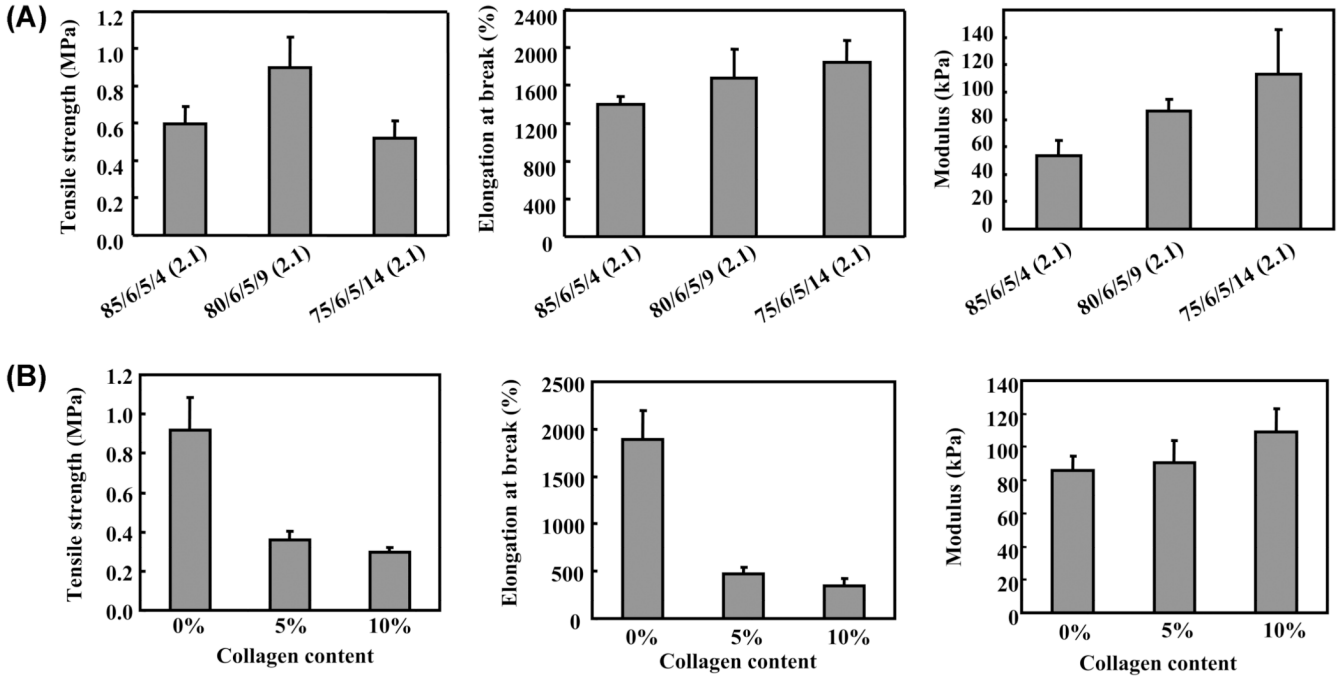


Figure 6.

Effect of polymer composition on mechanical properties at 37 °C. (A) effect of NIPAAm/HEMAPLA ratio, (B) effect of collagen content.

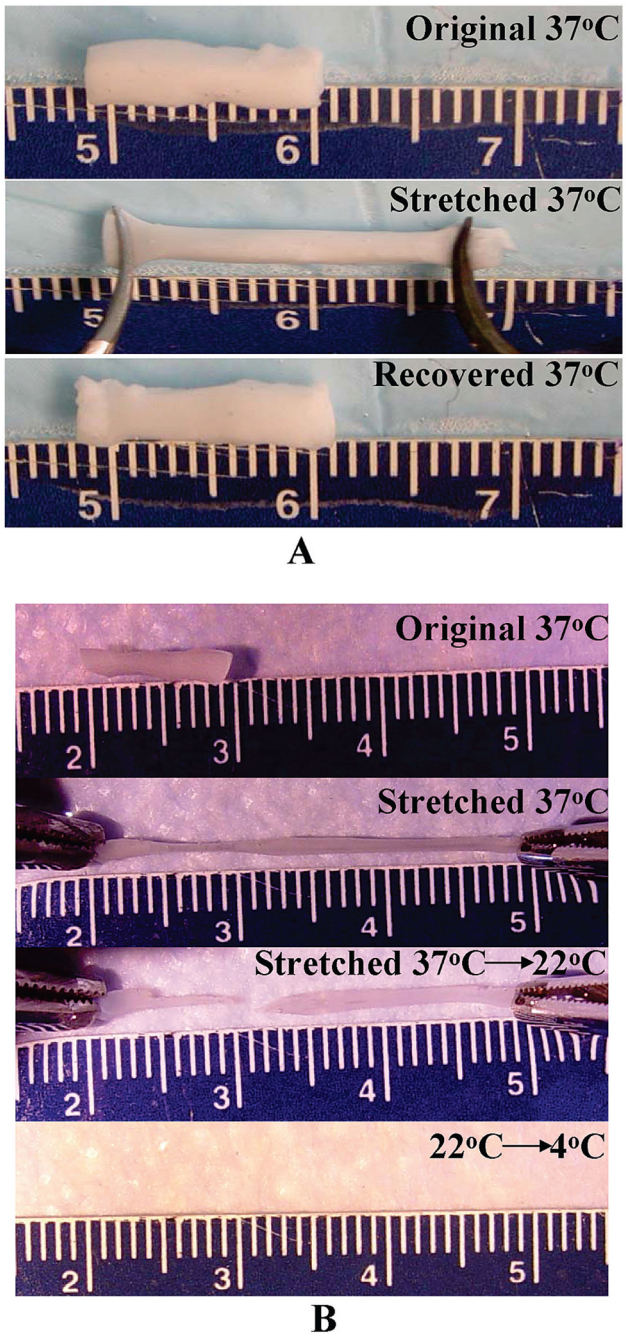


Figure 7.

Macroscopic images of the hydrogel in aqueous conditions (A) stretched and recovered at 37 °C, (B) stretched at 37 °C followed by a temperature decrease to 22 °C, whereupon the hydrogel fractured, and at 4 °C, where the fractured hydrogel completely dissolved.

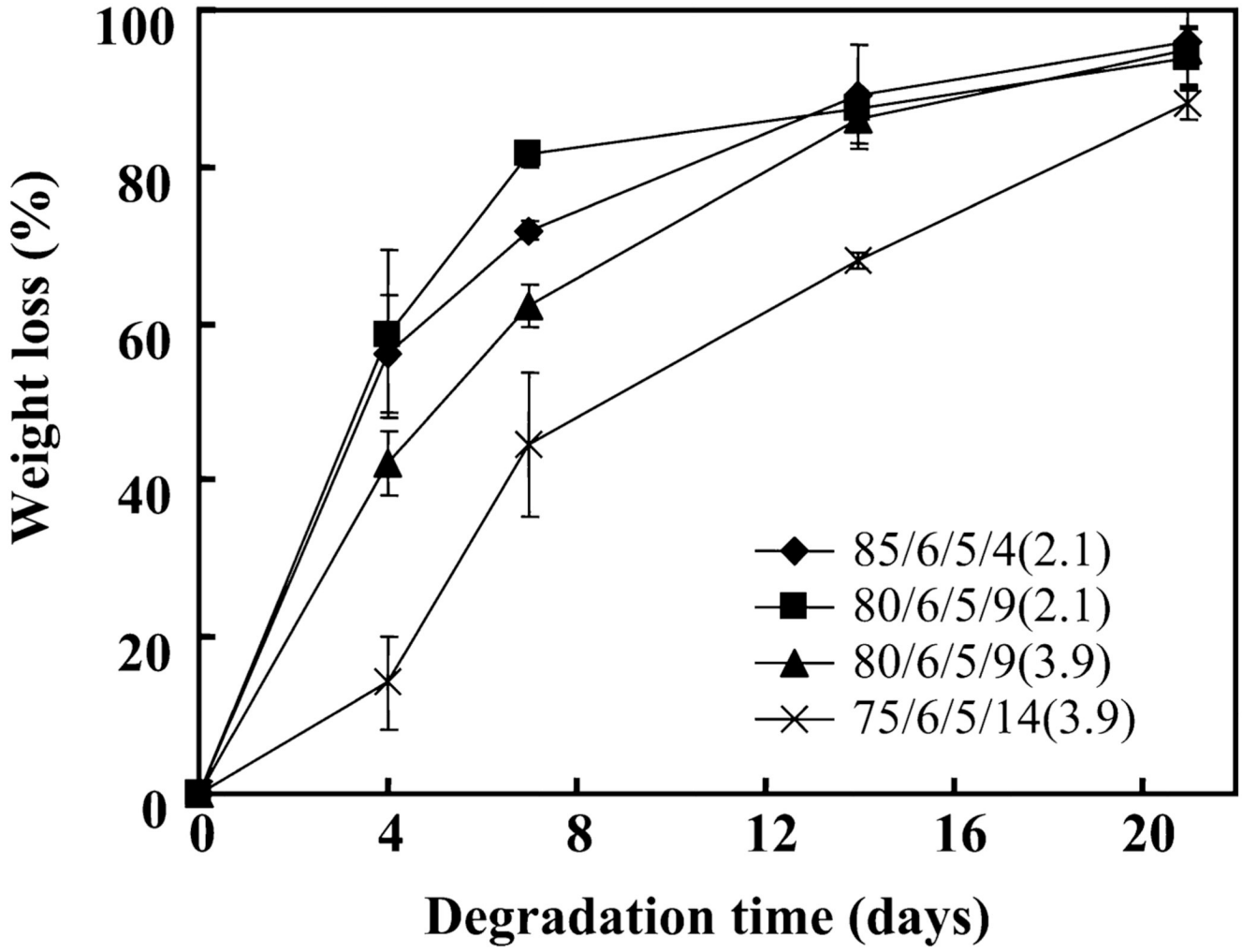


Figure 8.
Degradation of copolymer hydrogels in PBS at 37 °C.

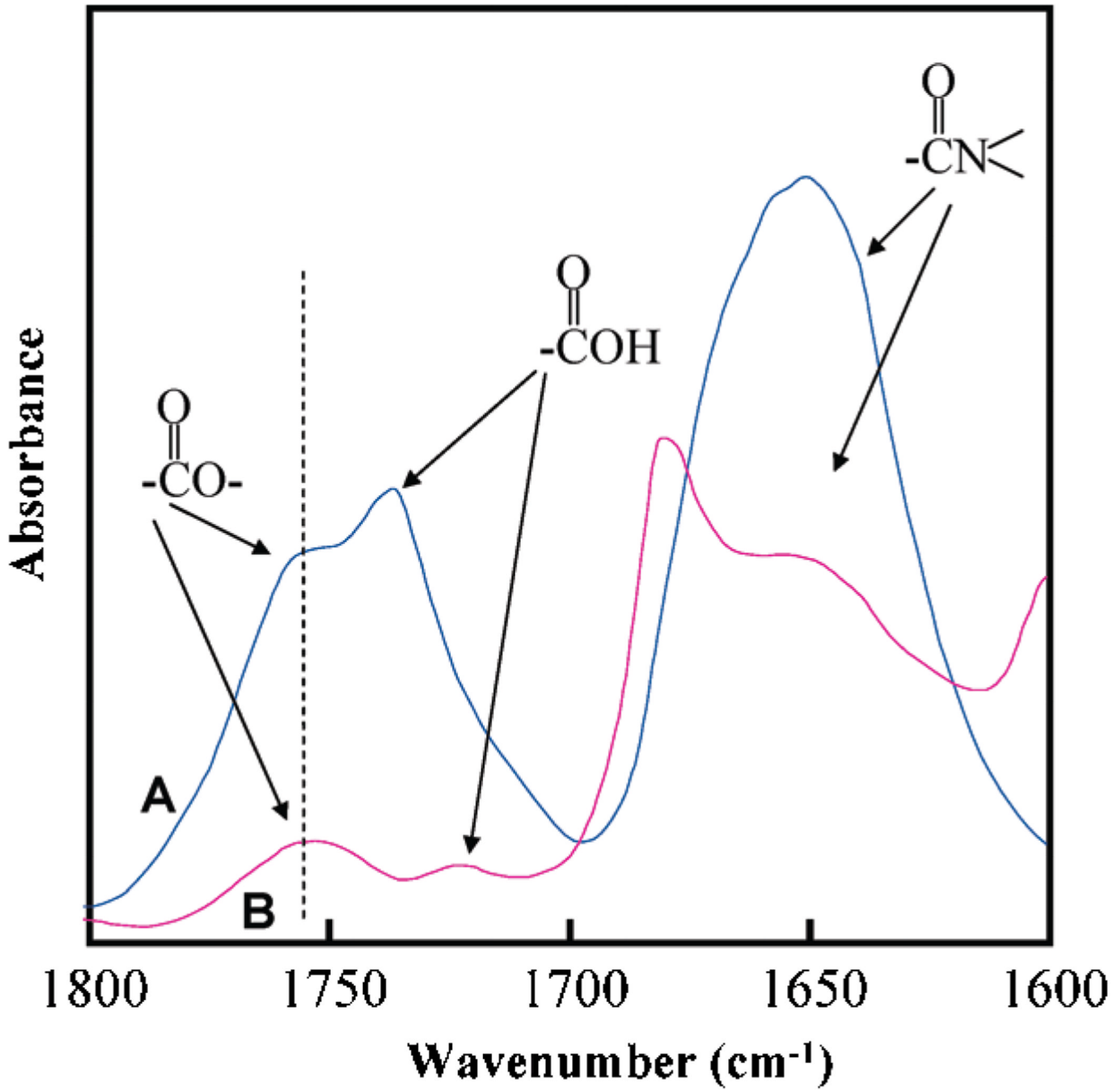


Figure 9.
FTIR spectra of poly(NIPAAm-*co*-AAc-*co*-NAS-*co*-HEMAPLA) before (A) and after hydrolysis in 1 M NaOH solution for 1 day (B).

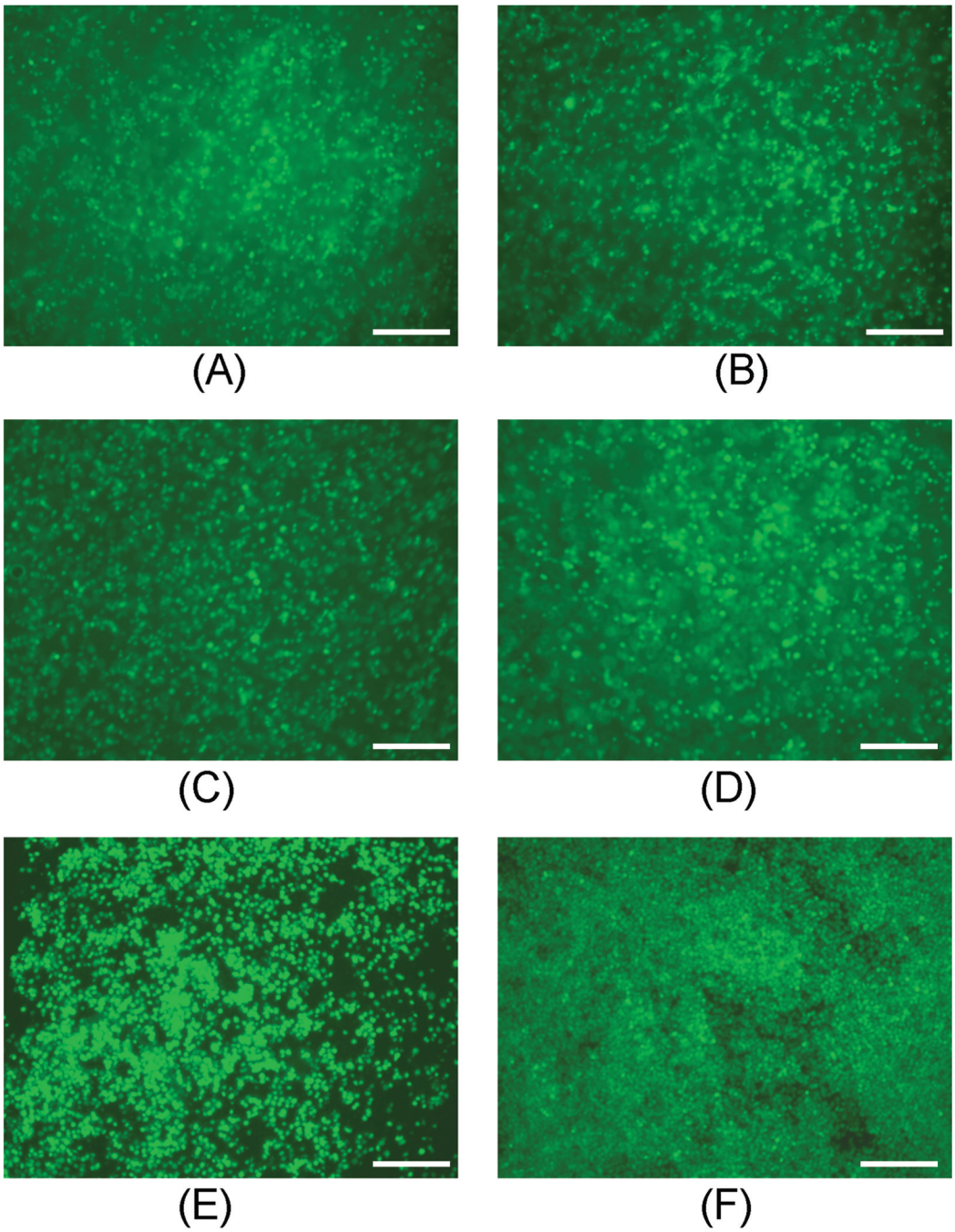


Figure 10.
Smooth muscle cell encapsulation in hydrogels without (A, C, and E) or with (B, D, and F) collagen. Cells were cultured in hydrogels for 0 (A and B), 3 (C and D), or 7 days (E and F). Scale bar) 200 μm.

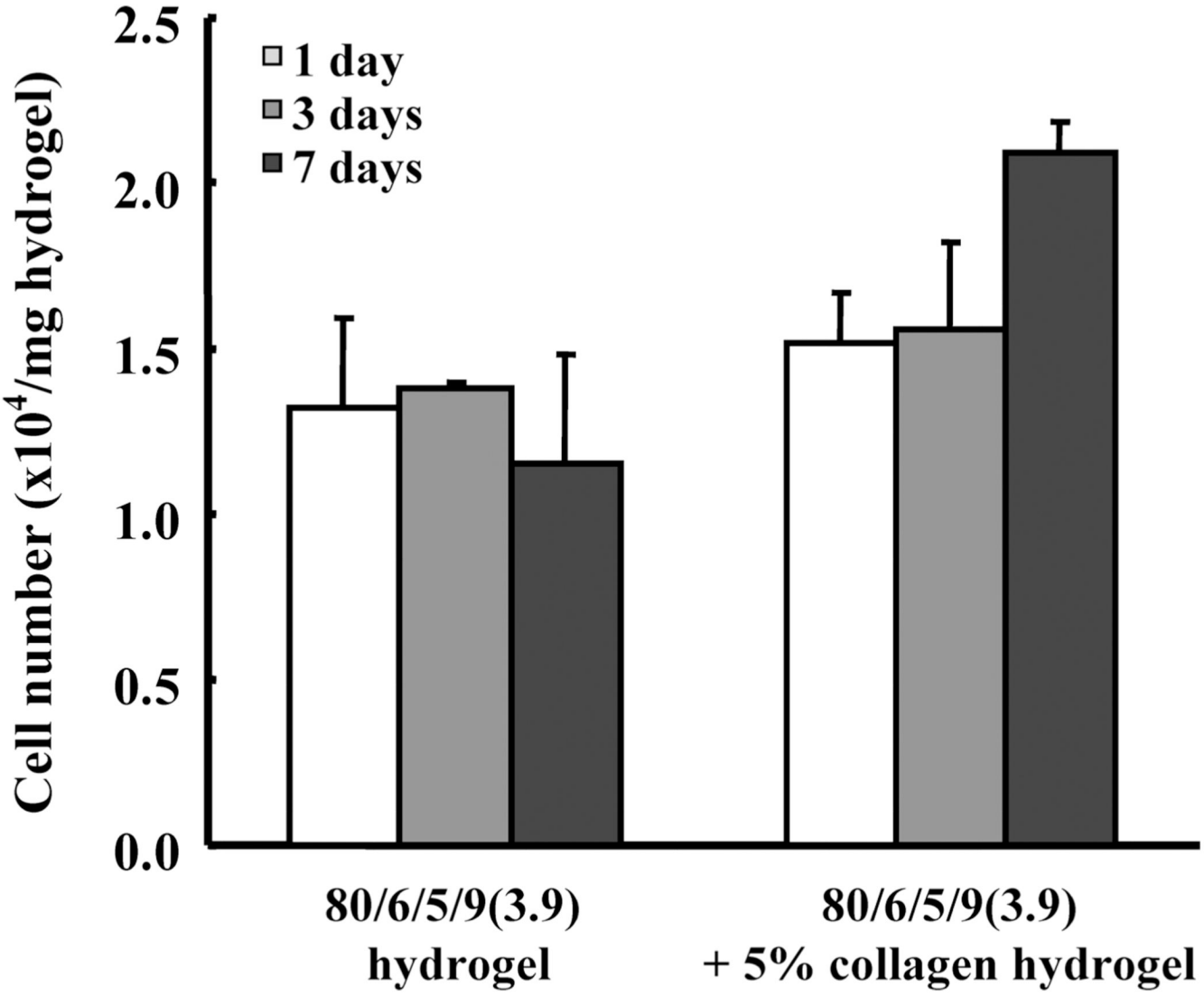
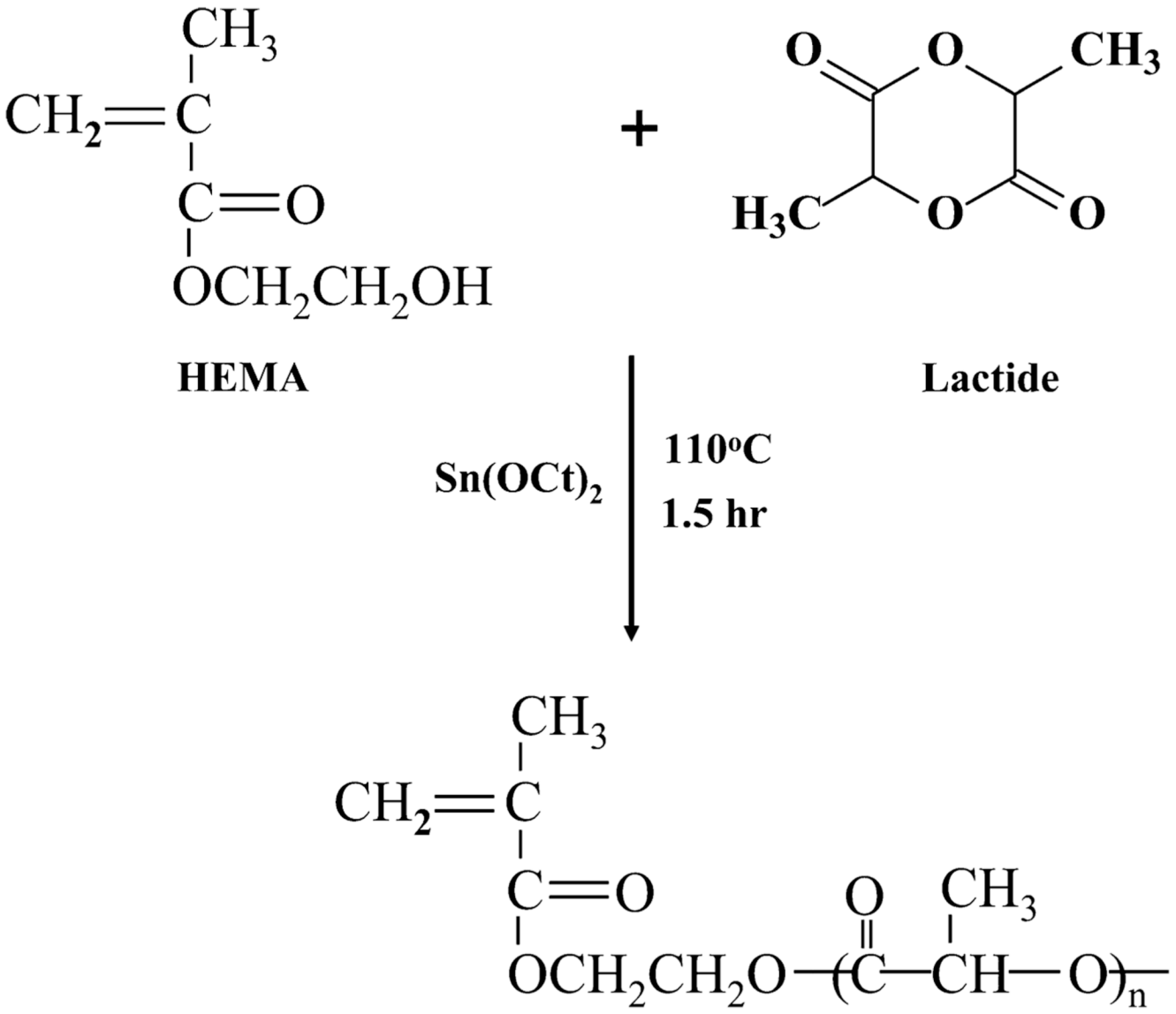
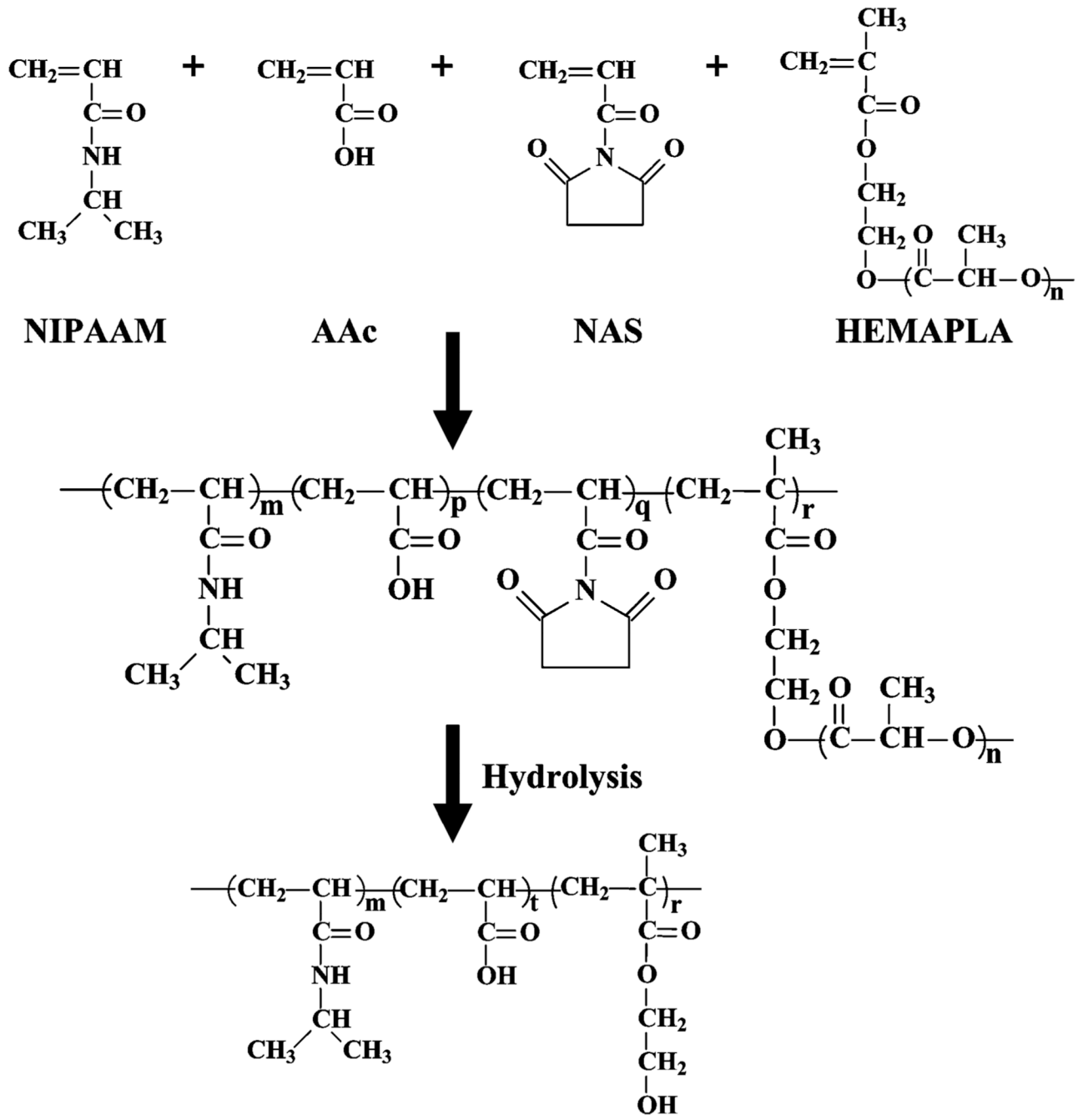


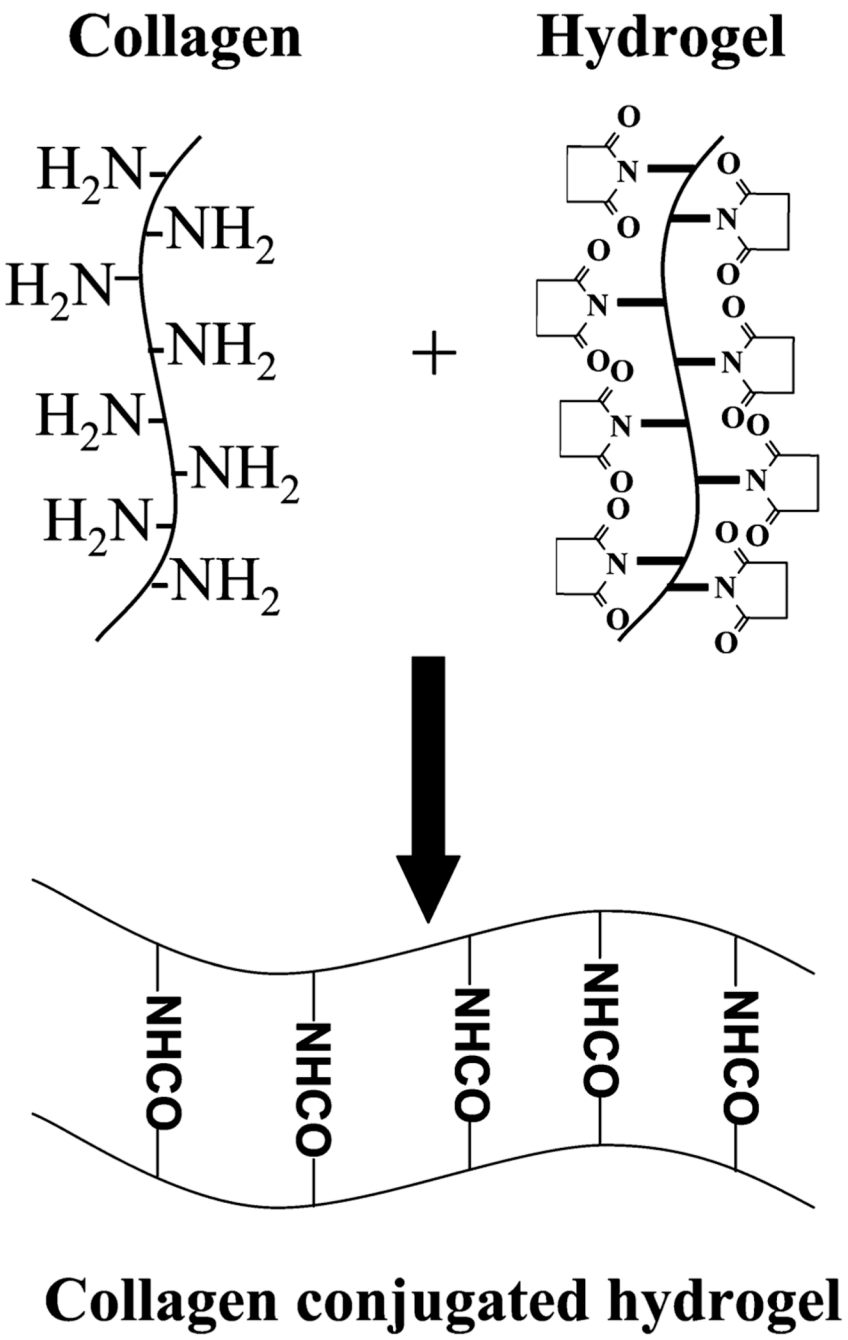
Figure 11.
Encapsulated smooth muscle cell growth in hydrogels with or without 5% collagen.



Scheme 1.
Synthesis of HEMAPLA Macromer.



Scheme 2.
Synthesis and Hydrolysis of poly(NIPAAm-*co*-AAc-*co*-NAS-*co*-HEMAPLA)



Scheme 3.
Collagen Conjugation with poly(NIPAAm-*co*-AAc-*co*-NAS-*co*-HEMAPLA)

Table 1

Composition and Molecular Weight of poly(NIPAAm-*co*-NAS-*co*-AAc-*co*-HEMAPLA)[a]

feed ratio[b]	composition	M_n
85/6/5/4(2.1)[b]	85/6.7/3.9/4.4	38672
85/6/5/4(3.9)[b]	85/6.9/4.0/4.1	32770
85/6/5/4(7.0)[b]	85/6.9/3.8/4.3	35175
80/6/5/9(2.1)[b]	80/7.5/4.2/8.3	33510
80/6/5/9(3.9)[b]	80/7.0/4.4/8.6	31124
75/6/5/14(2.1)[b]	75/7.3/4.7/13.0	32068
75/6/5/14(3.9)[b]	75/6.3/4.9/13.8	36089
80/11/5/4(2.1)[b]	80/11.4/4.2/4.4	29113
80/11/5/4(3.9)[b]	80/10.6/6.2/3.2	35919

[a] Determined by ^{1}H NMR. NAS = *N*-acryloxysuccinimide; AAc = Acrylic Acid.

[b] NIPAAm/AAc/NAS/HEMAPLA ratio. Data in parentheses represent the number of lactide units.

Table 2

Lower Critical Solution Temperature (LCST)

polymer p(NIPAAm-*co*-AAc-*co*-NAS-*co*-HEMAPLA)	after synthesis (°C)	after NaOH hydrolysis (°C)
85/6/5/4(2.1)	26.0	41.1
85/6/5/4(3.9)	25.8	41.4
85/6/5/4(7.0)	24.8	42.3
80/6/5/9(2.1)	24.0	41.8
80/6/5/9(3.9)	21.0	42.0
75/6/5/14(2.1)	19.4	41.2
75/6/5/14(3.9)	18.4	40.8
80/11/5/4(2.1)	26.1	60.6
80/11/5/4(3.9)	26.0	61.1

Table 3

Effect of Collagen Content on Hydrogel Formation[a]

polymer p(NIP AAm-*co*-AAc-*co*-NAS-*co*-HEMAPLA)	collagen content (%)			
	0	5	10	20
85/6/5/4(2.1)	+	+	+	−
85/6/5/4(3.9)	+	+	+	−
85/6/5/4(7.0)	+	+	+	−
80/6/5/9(2.1)	+	+	+	−
80/6/5/9(3.9)	+	+	+	−
75/6/5/14(2.1)	+	+	+	−
75/6/5/14(3.9)	+	+	+	−
80/11/5/4(2.1)	+	+	+	−
80/11/5/4(3.9)	+	+	+	−

[a] + Gelation (formed hydrogel), − Precipitation (did not form hydrogel).