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Injectable pH- and temperature-responsive poly(N-isopropylacrylamide-co-propylacrylic acid) copolymers for delivery of angiogenic growth factors

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Abstract

A new sharply pH- and temperature-responsive hydrogel system was designed for delivering drugs to regions of local acidosis, as found in wound healing, tumor sites, or sites of ischemia. The reversible addition fragmentation chain transfer (RAFT) polymerization technique was used to synthesize copolymers of N-isopropylacrylamide (NIPAAm) and propylacrylic acid (PAA) with feed ratios of PAA between 0 and 20 mol %. The pH-responsive viscoelastic properties of these materials as a function of pH and temperature were quantified by rheometry. At physiologic pH (7.4) and 5 wt %, the polymer did not form gels, but rather remained soluble at temperatures as high as 50 °C. At lower pH values (pH ca. 5.5 and below) the polymer was liquid at 20 °C but exhibited a sol-gel phase transformation with increasing temperature and existed as a physical gel at 37 °C. Incorporation of the hydrophobic monomer, butyl acrylate, into the random copolymer raised the pH of gel formation to greater than 6.0 at 37 °C. Drug loading studies demonstrated that p(NIPAAm-co-PAA) hydrogels are able to maintain the bioactivity of basic fibroblast growth factor following storage in hydrogel for 40 h and can provide sustained pH-dependent release of vascular endothelial growth factor over a period of at least three weeks. This hydrogel system will thus gel at controllable acidic pH values upon injection, and is designed to undergo gradual dissolution as it performs its drug delivery function and the ischemic site returns to physiological pH.

Keywords

pH-sensitive; temperature-sensitive; hydrogel; injectable

Introduction

Polymers that form physical hydrogels in response to clinically-relevant stimuli, such as changes in temperature or pH, have significant potential for use as injectable depot drug delivery systems.^{1–5} Their high water content provides good biocompatibility, and the lack of chemical cross-links facilitates eventual elimination from the body.² Thermoreversible gelation has also been studied extensively in a variety of polymer systems, including Pluronics®,⁶ random copolymers of N-isopropylacrylamide and acrylic acid (p(NIPAAm-co-AA)),⁷ triblock copolymers of poly(ethylene glycol) and poly(lactic acid-co-glycolic acid) (PLGA-PEG-PLGA),⁸ and some natural polymers.² These materials exhibit a sol-gel transition when the temperature is increased above the gelation temperature.

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Recently, a number of researchers have investigated the use of pH as a stimulus for reversible gelation in polymeric systems.^{9–14} The pH-sensitive system could facilitate drug delivery to regions of local acidosis, including sites of infection,¹⁵ neoplasia,¹⁶ or ischemia.^{17, 18} The incorporation of carboxylic acid-derived monomers, such as acrylic acid (AA),^{7, 19–21} or methacrylic acid (MAA),^{22, 23} has been used to impart pH-sensitivity in a variety of copolymers. With pKa values lower than the physiologic pH of 7.4 (polyAA 24 pKa ~ 4.28, polyMAA²⁵ pKa ~ 5), these polymers can be designed to target acidic regions. However, under physiological conditions, the very low pKa values of polyAA and polyMAA generally limit use of these polymers for drug targeting to very low pH systems such as the stomach. Our group has reported recently on polymers containing propylacrylic acid (PAA).^{26–28} The longer alkyl segments on polyPAA raises the carboxylate pKa (relative to polyAA and polyMAA) and facilitates sharp phase transitions at pH values greater than pH 6.0.²⁸

Hydrogel systems that are sensitive to both pH and temperature are desirable because they offer more than one mechanism by which the gel properties of a system can be tuned. For example, chemically-crosslinked gels composed of p(NIPAAm-co-alkyl(acrylic acid)) exhibit changes in swelling behavior in response to both pH and thermal stimuli.^{29–31} By varying the MAA content in p(NIPAAm-co-MAA) crosslinked gels, Brazel and Peppas²⁹ observed changes in the lower critical solution temperature (LCST) and equilibrium swelling ratio. Similarly, comb-type grafted p(NIPAAm-co-AA) gels exhibited changes in their swelling behavior in response to changes in both pH (2.0 vs. 7.4) and temperature.³⁰ Kim and Healy³¹ developed temperature-responsive p(NIPAAm-co-AA) gels with proteolytically-degradable crosslinks that provide a controlled degradation mechanism. In another study, Lee and Vernon³² prepared a thermoreversible gel containing NIPAAm, acrylic acid, and 2-hydroxyethyl methacryl lactate (HEMA-lactate) that could be hydrolyzed, allowing the polymer to be cleared.

Our group recently characterized the pH and temperature response of poly(N-isopropylacrylamide-co-propylacrylic acid) (p(NIPAAm-co-PAA)) in dilute solutions.²⁸ P(NIPAAm-co-PAA) exhibited sharp transitions as determined by the LCST in the pH range of 5–6.²⁸ The reasons for the p(NIPAAm-co-PAA) phase change are two-fold: first, protonation of the carboxylic acid group at low pH renders the PAA component more hydrophobic in a reversible fashion, and second, increasing temperature stimulates the phase separation tendency of the pNIPAAm component. The extra hydrophobicity of the alkyl propyl segment of PAA was also interesting to compare to the previously studied alkylacrylic acid monomers (i.e. acrylic acid and methacrylic acid). In this study, we sought to develop a system that undergoes sharp, reversible gelation at intermediate acidic pH values (~pH 5–6) but remains soluble at normal physiologic pH (7.4). This pH response could first promote gel formation in diseased tissue exhibiting local acidosis, and second, promote polymer dissolution and elimination once the tissue has returned to normal physiologic pH. Such a system could be useful in providing sustained delivery of therapeutic molecules to regions of ischemia,¹⁷ such as in therapeutic angiogenesis that has the potential to promote healing of ischemic tissue.³³

Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received unless otherwise described. N-isopropylacrylamide (NIPAAm) was recrystallized from hexane. 2,2'-Azobisisobutyronitrile (AIBN) was recrystallized from methanol. Propylacrylic acid (PAA) was synthesized as shown previously.³⁴ Deuterated dimethylsulfoxide (DMSO) was obtained from Cambridge Isotope Laboratories (Andover, MA). 2-

dodecylsulfanylthiocarbonylsulfanyl-2-methyl propionic acid (DMP) was obtained a gift from Drs. Charles L. McCormick (University of Southern Mississippi) and John Lai (Noveon Company). Dulbecco's phosphate buffered saline (DPBS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), CyQUANT NF Cell Proliferation Kit and BODIPY-FL hydrazide were purchased from Invitrogen (Carlsbad, CA). PD-10 columns were purchased from GE Healthcare (Piscataway, NJ). Basic fibroblast growth factor (bFGF) was provided as a gift from Dr. Charles Murry (University of Washington) and Scios Inc. (Mountain View, CA). C2C12 mouse myoblasts from ATCC (Manassas, VA) (obtained as a gift from Dr. Albert Folch, University of Washington) were used between passage numbers 7 and 16. Vascular endothelial growth factor (VEGF) enzyme-linked immunosorbent assay (ELISA) DuoSet kit was purchased from R&D Systems (Minneapolis, MN).

Synthesis of p(NIPAAm-co-PAA)

Poly(NIPAAm-co-PAA) (Scheme 1) was prepared by RAFT polymerization as previously described²⁸ with DMP as the chain transfer agent (CTA), AIBN as the initiator, and 50 w/w % methanol as the solvent unless noted otherwise. Solutions were purged with nitrogen for 30 min and then allowed to polymerize at 60 °C for 17–42 h. After polymerization, the methanol was evaporated with a stream of dry air. The polymers were then dissolved in acetone and precipitated twice into ethyl ether. After drying the product under high vacuum for 24 h, the polymer yields were determined gravimetrically.

Polymer Characterization

Molecular weights were quantified by gel permeation chromatography (GPCmax VE2001, Viscotek, Houston, TX) using a single column. HPLC-grade dimethylformamide (DMF) containing 0.01 mol/L LiBr was used as the eluent. Molecular weights of p(NIPAAm-co-PAA) were calculated by comparison to a series of poly(methyl methacrylate) standards. Copolymer compositions were determined by proton nuclear magnetic resonance spectroscopy (¹H-NMR) by comparing the peak area of the NIPAAm isopropyl C-H signal at 3.9 ppm with the total peak area between 0.7 and 2.2 ppm (encompassing the remainder of C-H protons).

Polymer Sample Preparation

Stock solutions of each of the polymers were prepared by dissolving the polymer overnight in phosphate buffer (ionic strength 0.15 mol/L) at 4 °C. The pH values of the initial stock solutions were variable because in some preparations, small amounts of 1N NaOH were added (prior to pH adjustment) to the buffer solution to promote polymer solubility. Once fully dissolved, the concentration of the polymer samples was then adjusted to 5 wt % in phosphate buffer. The pH of the solutions was measured with an Accumet 950 pH/ion meter (Fisher Scientific, Hampton, NH) then adjusted as needed with small amounts of cold 1N NaOH or 1N HCl. Samples were prepared on ice to maintain polymer solubility over the entire pH range.

Gel Experiments

The tube inversion method was used to visually assess the sol-gel transition. Samples were considered a gel if vial could be inverted without flow for 30 seconds. One mL of a 5 wt % polymer solution at the appropriate pH value was added to a 4-mL screw capped vial. The solution temperature was then raised incrementally from 20 °C to 50 °C, allowing sufficient time for equilibration.

Rheology

Rheological measurements were performed on an AR-G2 rheometer (TA Instruments, New Castle, DE). One-mL samples were run in parallel plate configuration using a 40-mm stainless steel plate at a gap distance of 750 μm . An oscillatory strain sweep from 0.1–100% strain at a constant frequency of 1 Hz was performed to determine the linear viscoelastic region. In a typical experiment, values for constant strain and frequency were 2% and 1 Hz, respectively. Temperature ramps were performed to obtain values for the storage and loss moduli (G' and G'' , respectively) at a rate of 1°C/min to allow sufficient equilibration of the sample temperature. A solvent trap was used to prevent evaporation of water from the sample. A gel was identified when $\tan(\delta) \leq 1$ (where δ is the phase angle, and $\tan(\delta) = G''/G'$). In runs where there was rapid syneresis preventing the G' and G'' curves from crossing (due to slippage), the phase change was defined when the G' curve exhibited a rapid increase in slope.

bFGF Storage

P(NIPAAm-co-PAA) (37 kDa, 17 mol % PAA in polymer) was prepared as described above in phosphate buffered saline for bFGF storage. Aliquots (100 μL) containing 10 $\mu\text{g/mL}$ of bFGF (18 kDa), 10 mg/mL bovine serum albumin (BSA) (to prevent adsorption to tube walls), 10 $\mu\text{g/mL}$ heparin (to stabilize bFGF) and 5 wt % polymer were pipetted into 0.6 mL polypropylene microcentrifuge tubes then heated to 37 °C to promote gel formation. Samples were prepared in triplicate and incubated at 37 °C for 40 h then were immediately frozen and stored at –20 °C until further use.

Cell culture and bFGF Bioactivity

C2C12 mouse myoblasts were maintained in DMEM with 20% FBS, 100U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin (37 °C, 5% CO_2) and split every 2–3 days. Cells were plated 1500 cells/well in 96-well plates and allowed to attach overnight in 20% FBS. Cells were rinsed twice with DPBS then incubated with serum-free DMEM for 24 h. Basic FGF that had previously been stored for 40 h in p(NIPAAm-co-PAA) hydrogel (described above) was prepared at varying concentrations between 0–10 ng/mL bFGF ($n = 6$ per concentration) in DMEM containing 2% FBS and 10 $\mu\text{g/mL}$ heparin. Serum-free media was aspirated and replaced with bFGF-containing media then allowed to incubate for 72 h. The day before assay measurement, a standard curve was generated by adding cells of known density (between 0 – 40,000 cells/well) to the 96-well plate in triplicate and allowing cells to adhere overnight. Cells were quantified using CyQUANT NF Cell Proliferation Kit according to the manufacturer's instructions.

Fluorescence Labeling of P(NIPAAm-co-PAA)

P(NIPAAm-co-PAA) (37 kDa, 17 mol % PAA in polymer) was fluorescently labeled with BODIPY-FL hydrazide. P(NIPAAm-co-PAA) (100 mg, 2.7 μmol polymer, containing 0.46 μmol PAA groups) and dicyclohexylcarbodiimide (DCC, 1 mg, 3.27 μmol) were dissolved in dimethylformamide (DMF). BODIPY-FL hydrazide (1 mg, 3.3 μmol) was added to the DMF/polymer solution. The solution was reacted at room temperature overnight in the dark. Polymer was purified by precipitation into anhydrous ethyl ether and purification on a PD-10 desalting column.

Hydrogel Dissolution and VEGF Release Experiments

Non-fluorescent p(NIPAAm-co-PAA) (37 kDa, 17 mol % PAA in polymer) dissolved in phosphate buffered saline was doped with p(NIPAAm-co-PAA)-BODIPY with a weight ratio of 50:1 by bulk mixing. Aliquots (200 μL) containing 1 $\mu\text{g/mL}$ VEGF (45 kDa) and 5 wt % polymer were pipetted into 1.5 mL polypropylene microcentrifuge tubes then heated to

37 °C to promote gel formation. One mL aliquots of release medium (DPBS containing 1 mg/mL BSA, adjusted to pH 5, pH 6, or 7.4) were added to each tube. Samples were prepared in triplicate and incubated at 37 °C in a water bath, under agitation at 100 rpm. At days 1, 4, 7, 14, 21, and 28 after sample preparation, 400 μ L aliquots of release medium were removed and replaced with fresh release medium. The sampled release medium was stored in polypropylene tubes at -20 °C until further use. Hydrogel erosion was determined by measuring the fluorescence intensity (500 nm excitation wavelength, 511 emission wavelength) with a fluorescence microplate reader (Tecan Safire2, Switzerland). A standard curve of fluorescence intensity vs. known polymer mass was prepared at each time point to quantify the mass of eroded polymer. Released VEGF was quantified by a sandwich ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, a monoclonal antibody to VEGF was used to capture VEGF on a 96-well plate. A polyclonal antibody to VEGF conjugated to horseradish peroxidase (HRP) was then used to detect VEGF, using tetramethylbenzidine as a substrate for HRP. VEGF concentrations were quantified by measuring the absorbance at 450 nm in each well with a microplate reader (Tecan Safire2, Männedorf, Switzerland) then comparing these measurements to a standard curve of absorbance vs. VEGF concentration.

Results and Discussion

Polymer Characterization

The copolymer compositions, MWs, and polydispersity index (PDI) values for copolymers synthesized by RAFT in this study are shown in Table 1. Copolymerizations conducted in the presence of the RAFT chain transfer agent (CTA) DMP resulted in well-defined polymers as evidenced by the low PDIs and controlled molecular weights, in agreement with Yin et al.²⁸ The discrepancy between theoretical and experimental molecular weights is likely due to formation of dead chains at longer reaction times due to coupling reactions.³⁵ Because there is a decrease in the number of polymer chains available to participate in RAFT polymerization at later time points, the measured experimental molecular weight of remaining propagating chains is greater than that predicted by theoretical models. In addition, the use of a polymer standard (PMMA) to calculate experimental molecular weights by gel permeation chromatography (GPC) may have also introduced some error into the calculated experimental molecular weights.

Rheology

The storage modulus (G') and loss modulus (G'') were measured as a function of temperature. Figure 1 shows an example of typical rheometry data obtained during the sol-gel transition. Images obtained from the inverted tube test are shown (which has previously been shown to correlate well with rheological data³⁶). In the liquid (sol) phase (Region 1) at low temperature, the polymer was completely dissolved in solution. Under these conditions, G'' was greater than G' which is consistent with a molecularly dissolved polymer solution. Between the LCST and the gel temperature (Region 2), the sample became increasingly cloudy by visual inspection but did not form a gel. The decrease in G' and G'' seen in this region is likely due to the inability of the rheometer to provide accurate measurements of the heterogeneous polymer sample. As the sample crossed the gelation temperature, there was a rapid increase of both G' and G'' , with $G' > G''$ indicating the formation of a viscoelastic gel (Region 3). Finally, at high temperatures, syneresis occurred (Region 4), whereby phase separation seen as exclusion of water from the gel led to a rapid decrease of the measured values for G' and G'' . This falling off of the G' and G'' curves can be explained by slippage of the plate with the water layer, and it is difficult to interpret numerical values for G' and G'' beyond this point. For the purposes of this study, we have focused on the values of G' as this represents the elastic-like properties of the gel (the G'' curve was omitted from

subsequent figures for clarity). In preliminary data not shown here, we observed that the behavior of p(NIPAAm-co-PAA) was affected by changes in ionic strength in a manner similar to that described previously for the homopolymer pNIPAAm.³⁷ Therefore, ionic strength was held constant at 0.15 mol/L to better mimic physiological conditions for all experiments.

Concentration-dependent gelation—The gel properties of p(NIPAAm-co-PAA) were dependent on concentration (Figure 2). At concentrations of approximately 5 wt % and above, gel formation was seen as a rapid increase in slope in the G' curve as well as lack of flow during the inverted tube test. Below approximately 3 wt %, gel formation did not occur at pH 5.0; however there was an intermediate phase change to the cloudy liquid phase, and often there was formation of a thin polymer film on the rheometer plate at high temperatures while the remainder of the sample remained a liquid. This is similar to data previously reported with p(NIPAAm-co-acrylic acid) where there was no gel phase observed for concentrations less than or equal to 3 wt %.⁷ Thus, there exists a threshold concentration for p(NIPAAm-co-PAA) below which gel formation does not occur.

Propylacrylic acid versus methacrylic acid—In order to determine if the sol-gel transition under conditions of moderate acidosis would occur if a less hydrophobic acidic monomer, such as methacrylic acid (MAA), were used instead of PAA, the G' vs. temperature behavior of p(NIPAAm-co-PAA19) (“PAA19” indicating 19 mol % PAA in polymer) was compared to p(NIPAAm-co-MAA20) (20 mol % MAA in polymer) (Figure 3). P(NIPAAm-co-PAA19) was sensitive to pH, with gel formation at values less than or equal to pH 5.5. In contrast, p(NIPAAm-co-MAA20) did not form a gel at pH 5.0 or higher, and even at pH values as low as 2.0 (data not shown), gel formation occurred only with significant syneresis.

These results suggest that the less hydrophobic methyl group on MAA does not provide sufficient hydrophobic interactions between polymer chains for gel formation, in contrast to the more hydrophobic propyl alkyl segment of PAA. This is in agreement with previous studies examining the pH response of polymers containing poly(methacrylic acid) (PMAA), poly(ethylacrylic acid) (PEAA) or poly(propylacrylic acid) (PPAA).^{20, 27, 38} More hydrophobic alkyl side chains increase the pKa of the carboxylic acid group. It is likely the elevated pKa of PAA in p(NIPAAm-co-PAA) compared to MAA in p(NIPAAm-co-MAA) facilitates gelation at higher pH values.

Molecular weight dependence—Changes in molecular weight (MW) of p(NIPAAm-co-PAA17 or 19) (all contained 10 mol % PAA in feed) between 15–37 kDa did not appear to have a significant effect on the gelation temperature for a given pH (Table 2). For example, at pH 4.5 and 5.0, all three MWs of p(NIPAAm-co-PAA) exhibited a nearly identical gelation temperature.

This lack of a MW effect is in contrast to Shim et al.¹⁰ who found fairly significant shifts in the temperature-pH phase diagram curves with relatively small changes in MW in a sulfonamide-containing triblock. However, the MW range studied here may have been too small to observe significant shifts in the sol-gel behavior for a random copolymer. Han and Bae⁷ observed gelation of p(NIPAAm-co-AA) at pH 7.4 (and 0.2 mol/L ionic strength) with a MW of 1,000,000 Da, while the largest polymer we studied was closer to 40,000 Da. In order to use a reversible hydrogel *in vivo*, shorter polymer lengths are desired to facilitate clearance of the delivery system.

Effect of Polymer Composition—Polymer composition had a significant influence on the behavior of the copolymers. Figure 4 depicts the sol-gel behavior of polymers

synthesized with varying feed compositions of PAA. The homopolymer of pNIPAAm underwent a phase change from a clear liquid to precipitate at approximately 31 °C but did not form a gel (data not shown). In contrast, p(NIPAAm-co-PAA8) (8 mol % PAA in polymer) (Figure 4a) was sensitive to pH changes, with a decreased gelation temperature at lower pH values. P(NIPAAm-co-PAA28) (28 mol % PAA in polymer) (Figure 4b) exhibited even more sensitive changes in the gelation temperature in response to incremental changes in pH values compared to p(NIPAAm-co-PAA8). The G' plateau observed with p(NIPAAm-co-PAA28) but not p(NIPAAm-co-PAA8) after gel formation suggests that polymers with higher PAA content form more stable gels.

In both p(NIPAAm-co-PAA8) and p(NIPAAm-co-PAA28), gel formation did not occur at pH values greater than approximately 6.0, as seen by the lack of a rapid increase in slope in the curves at pH 6.0. The pKa of p(NIPAAm-co-PAA) is approximately 6.0, depending on the relative concentrations of NIPAAm and PAA.²⁸ At pH 6.0, all polymer compositions studied do exhibit a cloud point,²⁸ but they remained in the cloudy liquid phase or precipitated out of solution without formation of a true gel. It is likely that at the pKa of the polymer and above, the percentage of ionized carboxylate groups is too high to allow gel formation. Gel formation occurs only when the temperature is high (above the gelation temperature) and the pH is low (below the pKa). The aggregation of the hydrophobic groups at high temperature and low pH likely stabilizes the gel structure. Furthermore, hydrogen bonds may form between the carboxylic acid group of PAA and the amide group of NIPAAm to stabilize the gel structure.

By varying the relative ratios of NIPAAm and PAA in a random copolymer of p(NIPAAm-co-PAA), it was possible to tune the pH and temperature at which gel formation occurred (Figure 5). As pH decreased, the gelation temperature also decreased for a given composition. These trends followed the LCST data of dilute solutions for this polymer published previously,²⁸ although conditions of increased hydrophobicity (higher temperatures or lower pH values) were required to reach the gelation temperature than to reach the cloud point.

Incorporation of hydrophobic monomer to tune pH-responsive gelation

behavior—The ability of p(NIPAAm-co-PAA) to form a gel depends upon the pH of the polymer solution. As described above, p(NIPAAm-co-PAA) does not form a gel at pH 6, likely due to insufficient hydrophobic aggregation above the pKa of the polymer. Clinical applications may require the formation of a gel at or above pH 6. In order to tune the pH-responsive gelation behavior, we incorporated the hydrophobic monomer, butyl acrylate (BA), into the random copolymer to form p(NIPAAm-co-PAA-co-BA), under the hypothesis that increased hydrophobic content would shift the pKa and facilitate gelation at higher pH values. As seen in Figure 6, incorporation of BA (10 mol % in the feed) into the polymer facilitated gel formation at approximately 21 °C at pH 6. In contrast, p(NIPAAm-co-PAA) with the same mol % of PAA in the feed but with no BA had a very low storage modulus and no visible gel formation at pH 6, even at temperatures up to 45 °C.

bFGF Bioactivity

Bioactivity of bFGF following 40 h storage in p(NIPAAm-co-PAA) hydrogel was quantified by a C2C12 cell proliferation assay. The density of C2C12 cells incubated with varying concentrations of bFGF (0.1–10 ng/mL) was significantly increased with evidence of a dose response after 72 h incubation compared to C2C12 cells incubated in media alone (Figure 7). These results demonstrate that p(NIPAAm-co-PAA) hydrogel storage does not adversely affect the bioactive properties of bFGF and support the feasibility of this polymer for use in protein drug delivery systems.

Hydrogel Dissolution

Rather than relying on hydrolysis or enzymatic degradation to facilitate elimination of the delivery system as others have proposed for a variety of biodegradable systems,¹ our system does not incorporate biodegradable components and instead relies on changes in the pH of the surrounding environment (i.e. restoration of physiological pH with tissue healing) to facilitate hydrogel dissolution and subsequent elimination. To study this *in vitro*, hydrogel dissolution was quantified with the aid of p(NIPAAm-co-PAA)-BODIPY (Figure 8a). Hydrogels stored in release buffer at pH 7.4 underwent dissolution of over 25% of the p(NIPAAm-co-PAA) hydrogel by 24 h, and were completely dissolved by day 4. In contrast, hydrogels stored in pH 6 release buffer showed only minimal dissolution by day 28. Furthermore, hydrogels stored in pH 5 release buffer remained largely intact at day 28. Thus, above the pKa of the polymer (pKa ~ 6), the hydrogel readily redissolves into the surrounding solution, even at 37 °C. In contrast, at or below the pKa of the polymer, the hydrogel does not readily redissolve into the surrounding fluid, which enables it to act as a drug depot system under conditions of local acidosis.

The ability of this system to respond to environmental changes in pH allows the local milieu to dictate the time period of polymer elimination. Future studies will be necessary to evaluate if use of our drug delivery system to promote tissue healing *in vivo* will restore the local tissue pH to physiologic pH of 7.4 and promote dissolution and elimination of the polymer system. One limitation to this approach is if the dissolved polymer particles are too large to be effectively filtered by the kidneys to eliminate the polymer from the body. In this situation, incorporating a biodegradable component into the polymer could facilitate breakdown into smaller particle sizes for elimination.

VEGF Release

The release profiles of VEGF from a selected composition of p(NIPAAm-co-PAA) (Figure 8b) demonstrate that there are multiple factors controlling the rate of VEGF release from the hydrogel. At pH 7.4, the hydrogel completely dissolved within 4 days leading to rapid release of the VEGF stored in the hydrogel. In contrast, VEGF release from hydrogels stored in pH 5 and pH 6 release buffers occurred more slowly over the 28-day period. Although the copolymer was more soluble in pH 6 buffer than in pH 5 buffer (Figure 8a), VEGF released faster from the hydrogel in pH 5 buffer than in pH 6 buffer. As shown in Figure 8a, the majority of the copolymer was not in solution even after 28 days at both pH 5 and pH 6. Thus, an additional factor to take into consideration is the electrostatic interaction between VEGF (isoelectric point, pI = 8.5) and the copolymer. At pH 6, there were more anionic groups from the unprotonated carboxylate groups that were available to bind the cationic VEGF and hinder release. In contrast, at pH 5, there were fewer anionic groups available to bind VEGF due to protonation of the carboxylate groups at lower pH values. Thus the rate of release was controlled by a balance of polymer dissolution as well as electrostatic effects.

We found that the maximum cumulative release of VEGF from p(NIPAAm-co-PAA) was less than 100%. This was likely due to several factors, including instability of VEGF in buffer at 37°C, interactions of VEGF with polymer, loss of protein due to adsorption to tube walls, and dilutional error. In preliminary data, we found that for a given initial concentration of VEGF incubated in buffer at 37°C, the concentration of VEGF measured by ELISA decreased linearly with time. Therefore, to correct for VEGF instability, we divided the measured VEGF concentration in our hydrogel release experiments by the percent of VEGF recovered from buffer alone at each time point. However, this makes the assumption that VEGF exhibits similar stability in a polymer-free environment compared to one in which polymer is present. Furthermore, interactions between the cationic protein, VEGF, with the anionic polymer may have resulted in a conformational change in VEGF

that rendered it no longer able to bind to the monoclonal capture antibody to VEGF used in the ELISA. Finally, given the low working conditions of VEGF, it is possible that loss of VEGF due to adsorption to tube walls or dilutional error could explain our results.

Conclusions

Aqueous solutions of p(NIPAAm-co-PAA) random copolymers exhibit a sol-gel response within a physiologically-relevant range of pHs (~4.5 – 6.0) and temperatures (~20 °C – 50 °C). By varying the polymer composition or concentration in solution, one can tune the pH- and temperature-responsive behavior of the physical hydrogel. Addition of hydrophobic monomer increases the pH at which gelation can occur. P(NIPAAm-co-PAA) maintains the bioactivity of bFGF following storage at 37 °C and can provide pH-dependent sustained release of VEGF. These traits make it suitable for use in injectable depot drug delivery systems. The ability of this polymer to respond to multiple physiological stimuli offers potential in designing more intelligent drug delivery systems.

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Figure 1.

Typical rheological data (storage modulus, G' (■) and loss modulus, G'' (□)) of sol-gel phase change with accompanying images taken during tube inversion test. Region 1, polymer is completely soluble in liquid (sol) phase; region 2, some polymer aggregates begin to form but sample remains in a cloudy liquid phase; region 3, gel phase; region 4, syneresis of water from gel. Sample data from p(NIPAAm-co-PAA23) (23 mol % PAA in polymer, 27 kDa), pH 5.0, 5 wt % in phosphate buffer solution. Storage moduli (G') recorded in the linear viscoelastic region at constant strain (2%) and frequency (1 Hz) while temperature was increased at a rate of 1 °C/min.

Figure 2. Concentration-dependent sol-gel behavior of p(NIPAAm-co-PAA17)_{37kDa} (17 mol % PAA) in pH 5.0 phosphate buffered solution, 5 wt % (■), 1 wt % (Δ). Storage moduli (G') recorded in the linear viscoelastic region at constant strain (2%) and frequency (1 Hz) while temperature was increased at a rate of 1 °C/min.

Figure 3. Sol-gel behavior of (a) p(NIPAAm-co-PAA19) (19 mol % PAA, 27 kDa) (■) and (b) p(NIPAAm-co-MAA20) (20 mol % MAA, 27 kDa) (Δ) at pH 5.0, 5 wt % in phosphate buffer. Storage moduli (G') recorded in the linear viscoelastic region at constant strain (2%) and frequency (1 Hz) while temperature was increased at a rate of 1 °C/min.

Figure 4.

Storage moduli (G') of (a) p(NIPAAm-co-PAA8) (8 mol % PAA, 30 kDa) and (b) p(NIPAAm-co-PAA28) (28 mol % PAA, 15 kDa) at 5 wt % in phosphate buffer solution at pH 4.5 (■), 5.0 (Δ), 5.5 (◆), or 6.0 (○). Storage moduli (G') recorded in the linear viscoelastic region at constant strain (2%) and frequency (1 Hz) while temperature was increased at a rate of 1 $^{\circ}\text{C}/\text{min}$.

Figure 5.

Sol-gel phase diagram of p(NIPAAm-co-PAA) as a function of polymer propylacrylic acid (PAA) content (calculated from proton nuclear magnetic resonance data) at pH 5.0 (—■—) and 5.5 (---Δ---) 5 wt % in phosphate buffer. Molecular weight determined by gel permeation chromatography: 8 mol % PAA, 30 kDa; 19 mol %, 27 kDa; 23 mol %, 27 kDa; 28 mol %, 15 kDa. Storage moduli (G') and loss moduli (G'') recorded in the linear viscoelastic region at constant strain (2%) and frequency (1 Hz) while temperature was increased at a rate of 1 °C/min. Gel phase determined when $G''/G' \leq 1$.

Figure 6.

Sol-gel behavior of p(NIPAAm-co-PAA-co-BA) (28 kDa, 10 mol % PAA in feed, 10 mol % BA in feed) (■) and p(NIPAAm-co-PAA) (20 kDa, 10 mol % PAA in feed, 21 mol % PAA in polymer) (Δ) at 5 wt % in phosphate buffer solution at pH 6. Storage moduli (G') recorded in the linear viscoelastic region at constant strain (2%) and frequency (1 Hz) while temperature was increased at a rate of 1 °C/min.

Figure 7.

Bioactivity of bFGF following storage in p(NIPAAm-co-PAA17)_{37kDa} (17 mol % PAA) hydrogel for 40 h. C2C12 mouse myoblasts exposed to bFGF in media containing 2% fetal bovine serum for 72 h prior to quantification with the CyQUANT NF Cell Proliferation kit according to the manufacturer's instructions and quantified with a standard curve. Data shown are mean $\pm$ standard deviation, with sample size $n = 6$. * $p < 0.03$ vs. control (0 ng/mL bFGF), † $p < 0.05$ vs. 0.5 ng/mL bFGF.

Figure 8.

(a) Dissolution and (b) cumulative VEGF release from p(NIPAAm-co-PAA17)_{37kDa} (17 mol % PAA) hydrogel (5 wt % in DPBS). Release medium (DPBS + 1 mg/mL BSA) adjusted to pH 7.4 (■), pH 6 (Δ), or pH 5 (◆) was added on top of the hydrogel. Re-dissolved polymer quantified by measuring fluorescence in a microplate reader using a standard curve of fluorescence intensity vs. known fluorescent polymer concentration. VEGF was quantified by ELISA. Hydrogel completely dissolved by day 4 in pH 7.4 buffer, thus later time points were omitted from the graph. Concentrations are corrected for stability of VEGF as a function of buffer pH and time (as determined by VEGF recovery from buffer-only

samples, data not shown). Samples performed in triplicate; data shown are mean $\pm$ standard deviation.

Scheme 1.

Synthesis of p(N-isopropylacrylamide-co-propylacrylic acid) (p(NIPAAm-co-PAA)) by reversible addition fragmentation chain transfer (RAFT) polymerization.

Table 1

Characteristics of p(N-isopropylacrylamide-co-propylacrylic acid) and p(Nisopropylacrylamide-co-methacrylic acid) synthesized by RAFT copolymerization^a

Polymer	PAA feed (mol %)	PAA polymer ^b (mol %)	Polymerization time (h)	Yield (wt %)	DP ^c	Mn (theor) ^d (g/mol)	Mn (expt) ^e (g/mol)	PDI ^f
pNIPAAm	0	0	17	84	200	19 500	34 600	1.13
p(NIPAAm-co-PAA8)	5	8.1	17	90	200	20 900	30 300	1.12
p(NIPAAm-co-PAA17) ^g _{15kDa}	10	17	18	59	100	7 000	14 600	1.15
p(NIPAAm-co-PAA19) ^g _{27kDa}	10	19	24	69	200	16 000	26 600	1.13
p(NIPAAm-co-PAA17) ^g _{37kDa}	10	17	21	58	400	26 500	36 800	1.25
p(NIPAAm-co-PAA23)	15	23	42	71	200	16 400	26 500	1.13
p(NIPAAm-co-PAA28) ^h	20	28	42	67	200	15 600	15 400	1.37
p(NIPAAm-co-MAA20) ⁱ	10 ^h	20 ^h	16	90	200	20 300	26 500	1.14

^a Polymerization performed at 60 °C, 50 w/w% monomer in methanol, 2,2'-azobis(isobutyronitrile) (AIBN) as the initiator, 2-dodecylsulfanylthiocarbonylsulfanyl-2-methyl propionic acid (DMP) as the chain transfer agent (CTA), and [CTA]₀/[AIBN]₀ = 5 unless noted otherwise.

^b Amount of propylacrylic acid (PAA) in the polymer was determined by proton nuclear magnetic resonance (¹H-NMR).

^c DP, Targeted degree of polymerization = [monomer]₀/[CTA]₀.

^d Mn (theor), theoretical number average molecular weight = conversion * monomer molecular weight * DP.

^e Mn (expt), experimental number average molecular weight, determined by gel permeation chromatography (GPC) in dimethylformamide (DMF) containing 0.01 mol/L LiCl at 60 °C (poly(methyl methacrylate standard))

^f PDI, polydispersity index = Mw/Mn, where Mw is the weight average molecular weight.

^g Synthesized in DMF.

^h Synthesized in isopropanol.

ⁱ Methacrylic acid (MAA) used as monomer rather than PAA.

Table 2

Sol-gel transition temperature of p(NIPAAm-co-PAA17 or 19) (10 mol % in feed) at different molecular weights.^a

Mn^b (g/mol)	PAA (mol %)	pH 4.5	pH 5.0	pH 5.5
15 000	17	23.5 ± 0.2	30.5 ± 0.2	43.5 ± 0.2
27 000	19	24.1 ± 0.4	30.4 ± 1.0 ^c	46.9 ± 1.5
37 000	17	23.6 ± 0	29.9 ± 0.4	41.0 ± 1.5

^aPolymer prepared at 5 wt % in phosphate buffer. Values reported as mean ± standard deviation, with data collected in triplicate (n=3) unless noted otherwise. Storage moduli (G') and loss moduli (G'') recorded in the linear viscoelastic region at constant strain (2%) and frequency (1 Hz) while temperature was increased at a rate of 1 °C/min. Sol-gel transition temperature determined when G''/G' ≤ 1.

^bM_n, number-average molecular weight, experimentally-determined by gel permeation chromatography (GPC).

^cData averaged from a total of six samples (n=6).