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Tailoring the degradation rates of thermally responsive hydrogels designed for soft tissue injection by varying the autocatalytic potential

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Abstract

The ability to modulate the degradation properties of biomaterials such as thermally responsive hydrogels is desirable when exploring new therapeutic strategies that rely on the temporary presence of a placed scaffold or gel. Here we report a method of manipulating the absorption rate of a poly(N-isopropylacrylamide) ((poly(NIPAAm)) based hydrogel across a wide range (from 1 d to 5 mo) by small alterations in the composition. Relying upon the autocatalytic effect, the degradation of poly(NIPAAm-co-HEMA-co-MAPLA), (HEMA=2-hydroxyethyl methacrylate; MAPLA=methacrylate-poly(lactide) was greatly accelerated by adding a fourth monomer methacrylic acid (MAA) at no more than 2 mol% to obtain poly(NIPAAm-co-HEMA-co-MAPLA-co-MAA) (pNHMMj) where j reflects the MAA molar % in the reactant mixture. MAA residue introduction decreased the pH inside the hydrogels and in surrounding buffered solutions. Accelerated degradation positively correlated with MAA content in pNHMMj polymers, putatively by the accelerated cleavage of MAPLA residues to raise the transition temperature of the polymer above body temperature. Physical properties including thermal transition behavior and initial mechanical strength did not vary significantly with MAA content. A rat hindlimb injection model generally reflected the in vitro observation that higher MAA content resulted in more rapid degradation and cellular infiltration. The strategy of tuning the degradation of thermally responsive hydrogels where degradation or solubilization is determined by their polyester components might be applied to other tissue engineering and regenerative medicine applications where designed biomaterial degradation behavior is needed.

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Keywords

hydrogel; thermally responsive material; degradation; heart

Introduction

Appropriate biomaterial degradation behavior is essential for obtaining desired therapeutic outcomes in a variety of tissue engineering and regenerative medicine applications [1–3]. Biomaterial degradation theoretically should be aligned with the pace of cell infiltration and neo-tissue formation to allow the structural and functional integration of host tissue with tissue developed in the region of the implanted biomaterial. For example, rapid degradation of dermal grafts may favor a fibrotic response over a more constructive regeneration outcome [4]. Uncoordinated absorption of bone substitutes can cause mechanical mismatch and ultimately contribute to failure in load bearing [2,5]. In biodegradable arterial stent development, there is interest in assuring that the stent remains long enough to remodel the vascular wall in a stable fashion, but not much longer to avoid complications associated with a permanent foreign body in the vascular wall [6,7].

Thermally responsive hydrogels have been widely studied for their amenability to minimally invasive delivery in the realms of drug delivery, embolization therapy, cell delivery vehicles, tissue fillers and wound dressings [8–10]. More recently, intramyocardial injection therapy has been pursued using mechanically strong thermally responsive hydrogels to inhibit pathological ventricular dilatation after myocardial infarction, a major contributor to morbidity and mortality in ischemic cardiomyopathy [11–13]. As with other biomaterial applications where temporary mechanical support is the objective, the degradation behavior of thermally responsive hydrogels becomes a critical design consideration.

In seeking to control the degradation rate for a thermally responsive hydrogel that would be used in soft tissue injection, several other design criteria must be considered. Basic requirements are acceptably low levels of cytotoxicity of the polymer and degradation products and adequate thermal response to allow needle-based injection and stiffening in situ. In the role of cell carrier, one would want to maintain encapsulated cell viability. When tuning the degradation rate, one would ideally not impair the thermal sensitivity of the system or substantially alter the mechanical properties in the fluid or hydrogel state.

Polyesters, widely used as biodegradable polymers in general, have been utilized as a hydrophobic component in thermally responsive hydrogels to trigger dissolution and absorption of the hydrogels upon ester cleavage [14–16]. Polyester materials degrade faster under low pH conditions due to catalyzed hydrolysis [17]. The accumulation of acidic degradation products can lead to an autocatalytic effect, accelerating the hydrolysis of ester bonds [18–20]. It was anticipated that the autocatalysis effect could be employed to tune degradation rates across wide ranges. Thus, in this study the amount of acid in a thermally responsive copolymer backbone was varied to modulate the degradation rate of a poly(N-isopropylacrylamide) based hydrogel, poly(NIPAAm-co-HEMA-co-MAPLA) (pNHM, copolymerized with N-isopropylacrylamide (NIPAAm), 2-hydroxyethyl methacrylate (HEMA) and methacrylate-poly lactide (MAPLA)) [21]. The pendant hydrophobic MAPLA

sidechains become acidic units upon hydrolysis, resulting in a higher transition temperature and eventual solubility of the copolymer, without backbone cleavage. Different molar ratios of methacrylic acid (MAA) were incorporated into the copolymer to obtain poly(NIPAAm-co-HEMA-co-MAPLA-co-MAA) (pNHMMj). The effect of MAA on hydrogel degradation was studied as well as its effect on thermal and mechanical behavior. The cytotoxicity of pNHMMj hydrogels and their degradation products were evaluated and an in vivo degradation study of pNHMMj hydrogels was performed in a rat hindlimb injection model.

Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. N-isopropylacrylamide (NIPAAm) was purified by recrystallization from hexane and vacuum-dried. 2-Hydroxyethyl methacrylate (HEMA) was purified by vacuum distillation. Lactide, benzoyl peroxide (BPO), sodium methoxide (NaOCH_3), methacryloyl chloride, methacrylic acid (MAA) and other solvents were used as received.

Synthesis of methacrylate polylactide (MAPLA)

The synthesis of methacrylate polylactide was performed as previously described [21]. Briefly, NaOCH_3 /methanol was added to a lactide/dichloromethane solution to synthesize polylactide (HO-PLA-OCH_3) through ring-opening polymerization. MAPLA was synthesized by dropping methacryloyl chloride into a HO-PLA-OCH_3 /dichloromethane solution containing triethylamine. Dichloromethane was removed by rotary evaporation and the product was purified by flash chromatography to obtain MAPLA with yields of ~60%.

Synthesis of poly(NIPAAm-co-HEMA-co-MAPLA-co-MAA)

Poly(NIPAAm-co-HEMA-co-MAPLA-co-MAA) (pNHMMj) copolymers were synthesized from NIPAAm, HEMA, MAPLA and MAA by free radical polymerization. The feed ratios of NIPAAm, HEMA, MAPLA and MAA were $80/(10-j)/10/j$, where $j = 0, 0.5, 1, 2, 5, 10$ (Scheme 1). Monomers (0.066 mol) were dissolved in 180 mL of 1,4-dioxane containing 0.23 g BPO. The polymerization was carried out at 75°C for 20 h under argon protection. The copolymer was precipitated in hexane and further purified by precipitation from THF into diethyl ether and vacuum-dried, with yields of ~80%. Fluorescently labeled copolymers were synthesized using the same reaction conditions with fluorescein O-methacrylate added at a feed ratio of an additional 2%, with all of the other monomer molar ratios remaining constant and $j=0$. Fluorescently labeled hydrogels used in the in vivo study were prepared by dissolving 14.25 wt% unlabeled copolymer with 0.75 wt% labeled copolymer in PBS.

Characterization

^1H NMR spectra of pNHMMj were recorded with a 600 MHz Bruker spectrometer using CD_3Cl as a solvent. Molecular weight of the copolymers was determined by gel permeation chromatography (GPC, Waters Breeze System, Waters 1515 HPLC Pump, Waters 2414 differential refractometer). The copolymers were dissolved in THF at a concentration of 1 mg/mL and the GPC analysis was performed at 35°C. A poly(methyl methacrylate) standard

kit (Fluka, ReadyCal Set Mp 500-2700000) was used for molecular weight-elution volume calibration.

Rheology studies were conducted on a TA Instruments rheometer (AR2000) to observe viscosity changes in the hydrogels during the temperature induced sol-gel transition. The polymer solutions (15 wt% in PBS) were placed between two parallel plates. With a temperature sweep from 5 to 35°C and a heating rate of 5°C/min, the shear storage modulus G' and the loss modulus G'' were collected as a function of temperature at a fixed strain of 2% and a frequency of 1 Hz.

To measure the mechanical properties of the hydrogels, samples were incubated in a 37°C water bath for 24 h to reach a stable water content, and then the solid hydrogels were cut into rectangular strips 1 mm thick, 4 mm wide, and 25 mm long and then loaded in a water bath equilibrated to 37 °C. An ElectroForce 3200 Series II (Bose, Minnesota, US) equipped with a 2.5 N load cell was utilized to record the tensile stress-strain curve immediately after the samples were taken out of the water bath.

Hydrogel degradation was quantified by mass loss measurements. Hydrogels with known initial dry masses (~60 mg) were immersed into 6 mL of PBS at 37°C. At predefined time points over a 28 week period the hydrogels (n=3 each) were lyophilized and the relative mass loss was recorded. The pH of the supernatant during degradation was measured with an Accumet pH meter (Fisher Scientific, Waltham, MA). To compare the relative pH inside pNHMMj hydrogels, polymers were dissolved in PBS containing 2.5 μ M LysoSensor Yellow/Blue DND-160 pH-sensitive dye (Life Technologies, Grand Island, NY, US). After gelation, the excess fluid was removed and replaced with PBS. The hydrogels were allowed to stabilize for 1 d before being placed in a plate reader with excitation at 360 nm and emission intensities at 440 nm and 540 nm measured for the hydrogel surface and cross sections (cut and exposed). The cross sections were used to semi-quantitatively determine whether there was detectably lower pH in the hydrogel interior.

Cytotoxicity of degradation products

The cytotoxicity of the pNHMMj degradation products was assessed by measuring the relative metabolic activity of rat vascular smooth muscle cells (rSMCs) cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Life technologies) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and supplemented at 10% with hydrogel degradation solution, as previously described [21, 22]. The hydrogel degradation solution was prepared from incubation of the hydrogel in PBS. Culture medium with PBS added at 10% was used as a negative control. rSMCs were seeded at an initial density of 30,000/cm² and their metabolic activity was measured (n=6 each) using an MTS assay kit (Promega CellTiter 96 Cell Proliferation Assay). To qualitatively verify the results of the above test, cells were also observed under fluorescence microscopy after live/dead staining with a Promokine Live/Dead Cell Staining Kit.

Viability of cells encapsulated in pNHMMj hydrogels

rSMCs labeled with the live cell marker CellTracker Red CMTPX (Life Technologies, Grand Island, NY) were suspended in PBS at a density of 2×10^7 /mL. A total of 0.25 mL of

this cell suspension was then added into 1 mL of the hydrogel solutions at 4°C. The mixture was thoroughly mixed before being transferred into a 37°C water bath for gelation. The supernatant was removed and replaced with culture medium (DMEM supplemented with 10% FBS, and 1% penicillin/streptomycin). The medium was changed every 3 d. After 1 and 7 d of culture, samples were taken out and cut into 100 µm thick sections and observed directly under fluorescence microscopy (Eclipse Ti-U, Nikon Instruments). At 7 d another set of samples were cooled at 4°C to release the encapsulated cells and these released rSMCs were then cultured on TCPS for an additional 7 d to qualitatively assess their ability to proliferate. In a separate set of experiments, unstained rSMCs were encapsulated as described above and upon recovery from the hydrogels by cooling at 1 and 4 d were stained with trypan blue solution and the percentages of live cells were calculated by manual counting of multiple microscopic fields for 3 independent samples for each hydrogel type.

In vivo hydrogel injection studies

Adult female Lewis rats weighing 160–210 g were utilized in a protocol that followed the National Institutes of Health guidelines for animal care and that was approved by the University of Pittsburgh's Institutional Animal Care and Use Committee. Anesthesia was induced with 3.0% isoflurane inhalation with 100% oxygen followed by 1.5–2% isoflurane with 100% oxygen during procedure. Dermatotomy was performed to expose the inner thigh muscles on both legs. Single injections of 200–250 µL of hydrogel (fluorescently labeled or unlabeled) were made approximately 3 mm deep in the muscle bed. For each hydrogel, 6 injections in 6 legs were made (4 labeled, 2 unlabeled). Inner thigh muscles from 2 legs of the labeled groups were excised 3 min after injection. The muscles were incised to expose the hydrogels, and images were taken with a Dino-Lite (AM4113T-GFBW, AnMo Electronics, New Taipei City, Taiwan) under brightfield and fluorescence mode. After 21 d, rats were sacrificed and the inner thigh muscles encompassing the hydrogels were excised, images taken and the tissue was fixed in 10% formaldehyde for 3 d before embedding. H&E staining (for unlabeled hydrogels) and immunohistochemical staining (with labeled hydrogels) with monoclonal antibodies against CD68 (1:100, Abcam) was performed. Nuclei were stained with 40',6-diamidino-2-phenylindole (DAPI; 1:10000, Sigma). Microscopic images were taken under fluorescence microscopy and assessed with ImageJ.

Statistical analyses

For the paired comparisons of Figure 2, a paired t-test was employed. Where three or more groups were being compared, one-way ANOVA was employed with Tukey's test applied for specific comparisons. Results are presented as the mean with standard deviation. Statistical significance was defined as $p < 0.05$.

Results

MAA content in the pNHMMj hydrogels

The incorporation of MAA into the hydrogels was confirmed by NMR as the -COOH peak appeared at ~12 ppm on the ¹H spectrum, as shown in Figure 1a. The content of MAA in the copolymer calculated with peak areas shows a linear increase with the MAA feed ratio, (Figure 1b) although this content is ~40% lower than the MAA feed ratio in the reaction

system. This result was also confirmed with minor discrepancies from the NMR results by titrating the -COOH groups in the copolymer with HCl/NaOH for protonation/deprotonation (Figure 1b). GPC results showed that the molecular weights M_w of the pNHMMj copolymers were all between 22000 and 26000 g/mol.

Degradation and dissolution of pNHMMj hydrogels

The pH of the supernatant solution after gelation of the pNHMMj polymers in PBS buffer (15 wt% of copolymers) showed that with increased MAA content in the copolymer, the more acidic the initial degradation environment was for PLA side chains in this limited volume system, Figure 2. Measured immediately after changing PBS, the supernatant pH for all pNHMMj increased, and showed no significant difference compared to the pH of PBS. After stabilization for 24 h to allow diffusion, the supernatant pH of pNHMM0, pNHMM0.5 and pNHMM1 remained above 7.3, whereas the pH for pNHMM2 dropped below 7.1 (Supplemental Figure 1, pNHMM5 and pNHMM10 degraded too quickly for this measurement). After another cycle of PBS change and measurement after another 4 d, the pNHMM2 continued to be able to reduce the pH versus the other hydrogels. On the other hand, after gelation and placement in fresh PBS, followed by 24 h stabilization, the pH was lower in and on the surface of pNHMM2 hydrogel compared to pNHMM0, pNHMM0.5 and pNHMM1, as indicated by a pH-sensitive dye (LysoSensor, whose emission intensity ratio between 540 nm and 440 nm increases as pH decreases). In addition, the pH of the interior of the pNHMM2 hydrogel was lower than the pH on the surface. No significant differences were found among the other 3 polymer types (data not available for pNHMM5 and pNHMM10 due to rapid degradation).

The degradation rate increased significantly as MAA was added in increasing proportion into the polymer backbone, as shown by the weight loss profile of the hydrogels in PBS, Figure 3a. Without MAA, pNHMM0 needed over 5 mo to lose 50% weight in PBS, and the same loss required about 2 mo, 1 mo, 1 wk and 1 d for MAA containing copolymers with the MAA feed ratio at 0.5% (pNHMM0.5), 1% (pNHMM1), 2% (pNHMM2), 5% (pNHMM5) and 10% (pNHMM10), as shown in Figure 3b. The temporal weight loss profiles of the pNHMMj hydrogels share a similar shape, which begins with a slow weight loss stage, followed by an abrupt decrease in remaining weight. Furthermore, when incubated in PBS solution at pH 9.5, the abrupt weight loss for MAA10 was postponed for 1 d (Supplement Figure 2). Since pNHMM5 and pNHMM10 hydrogels were considered to degrade too quickly for potential in vivo applications, hydrogels with less MAA were selected as candidates for further evaluation.

Thermal and mechanical behavior of pNHMMj hydrogels

The transition temperature of MAA hydrogels in PBS did not shift significantly as the MAA content in the polymer backbone was increased. As shown in Figure 4a, the rapid increase in shear modulus G' of pNHMM0, pNHMM0.5, pNHMM1 and pNHMM2 occurred within $17.5 \pm 2.5^\circ\text{C}$, which represents the sol-gel transition as temperature rises. Accompanying this mechanical transition the copolymers were observed to become optically opaque and form white gels. The abrupt increase in shear modulus or viscosity required only a few sec at 37°C , hence when pNHMMj hydrogels were injected into PBS at 37°C , the response was

rapid. As shown in Figure 4b, solution pH did not significantly affect the transition temperature of pNHMMj hydrogels, especially in the weak acidic range.

The Young's modulus of pNHMMj hydrogels were all above 200 kPa and did not vary significantly with increasing MAA content (Figure 5), indicating that the degradation or absorption rate for the hydrogels was decoupled from their initial mechanical stiffness in tension when the MAA content was low.

Cytocompatibility of pNHMMj hydrogels and their degradation products

As shown in Figure 6a–d, round single cells were evenly distributed when rSMCs were encapsulated within the hydrogels. Using trypan blue to quantify viability, one day after encapsulation, over 85% cells were alive in pNHMM0, pNHMM0.5, pNHMM1 gels, while less than 70% cells survived in pNHMM2 (Figure 6i). Viability was ~85% in pNHMM0, pNHMM0.5, pNHMM1 gels 4 d after encapsulation, while viability was less than 60% in pNHMM2 (Figure 6i). Qualitatively assessing cells stained with a viability marker at the time of encapsulation showed that after 1 and 7 d culture, viable cells remained spread throughout the hydrogels (Figure 6e–h). Cells isolated at 7 d and cultured on TCPS for 7 d qualitatively showed an ability to proliferate (Supplement Figure 3).

The cytotoxicity of the degradation products of pNHMMj hydrogels were also tested. Live/dead staining showed that rSMC proliferation was not impeded by any of the copolymer degradation products: overall metabolic activity of the cultures during the 7 d increased, suggesting cell proliferation, and few dead cells were observed after recovery and culture on TCPS (Figure 7a–o). Slightly different from the findings from the encapsulation experiment, degradation products of the pNHMM2 hydrogel did not show higher cytotoxicity compared to its counterpart pNHMMj hydrogels (Figure 7p).

In vivo degradation

In vivo degradation and absorption of hydrogels was tested in rat hindlimb muscles. Hydrogels solidified immediately upon being injected into the muscles and formed distinct volumes, displacing the muscle tissue, as shown in the left columns of Figure 8. Fluorescent imaging revealed that the hydrogels formed well-defined regions, with minimal diffusion into the tissue. This effect was also demonstrated on immunohistochemical staining sections obtained from tissue immediately after injection (data not shown). After 21 d, the boundaries between injected hydrogels and the leg muscles were more diffuse compared to the time right after injection, particularly for pNHMM1 and pNHMM2, as shown in the right columns of Figure 8. Fluorescent signals could still be observed, showing that the hydrogels were not completely absorbed. However, the fluorescence intensities were weaker in all four groups compared to the initial status. Unabsorbed hydrogels could be identified in immunohistochemical sections in all four groups as irregularly shaped regions encompassed by dense cell populations (Figure 9a). For most of the polymers these regions of injection had minimal or no fluorescence, while for the pNHMM0 group this region was seen to have higher green fluorescence in large, continuous areas. The dense cell populations surrounding the polymers were CD68 positive and identified as macrophages (Figure 9a,b). For pNHMM0, pNHMM0.5 and pNHMM1 hydrogels, macrophages encompassed the materials,

however, did not appear to infiltrate into the polymer. For the pNHMM2 group, macrophages infiltrated into the injection site, separating the remaining hydrogel volumes. Similar observation could be made on H&E stained sections. Macrophages gathered and formed capsules around injected hydrogels as shown in Figure 9c. Macrophages can be found in some regions of unabsorbed pNHMM2 hydrogel, migrating from the border zone. This was not observed with the other 3 polymer types.

Discussion

Different strategies have been reported to modulate the degradation of thermally responsive hydrogels. For poly(ethylene glycol)-poly(ϵ -caprolactone)-poly(ethylene glycol) (PEG-PCL-PEG), poly(ethylene glycol)-poly(lactide-co-glycolide)-poly(ethylene glycol) (PEG-PLGA-PEG) and similar block copolymer systems, changing the molecular weight of individual blocks and the molecular weight ratio between hydrophobic and hydrophilic blocks results in changes in degradation rate [23, 24]. For thermally responsive systems that employ crosslinking, adjusting the crosslinking density is effective [25]. In poly(N-isopropylacrylamide) based hydrogels, the content of hydrolytic pendant groups has been shown to have a significant influence on both hydrogel degradation rate and thermal transition behaviors [26]. In other cases, introducing enzyme sensitive cleavage sites has proven an effective mechanism [27–29].

In this study, pNHMMj hydrogels were synthesized and displayed tunable degradation behavior in vitro and in vivo, supporting the design hypothesis that the addition of MAA as an autocatalyst for MAPLA hydrolysis would provide a method to accelerate the hydrogel dissolution. Manipulation of MAA content over a relatively small range resulted in widely varying degradation behavior, with 50% solubilization occurring in time frames from days to months, wider than what has been previously reported in the literature [21, 22, 30, 31]. The lower pH of the supernatant immediately after gelation and after a series of PBS/medium changes and stabilization steps indicated that the proton concentrations were higher in the environment for pNHMMj hydrogels with higher MAA content, as expected. This result also corresponds with the pH being lower inside and on the surface of pNHMM2 compared to other pNHMMj hydrogels having less MAA content, since greater MAA content would be the source of extra protons which decreased the supernatant pH. In addition, the pH appeared to be lower inside pNHMM2 compared to the surface of the hydrogel, suggesting diffusion-driven depletion of protons towards the surface of the hydrogel. The four pNHMMj hydrogels studied all experienced an abrupt mass loss at different times, indicating a rapid increase in hydrolysis rate and polymer hydrophilicity. This can be explained with the hindered diffusion of protons. As the proton concentration slowly builds up in the hydrogels, the autocatalytic effect would become stronger, leading to faster accumulation of cleaved lactic acid molecules and MAA residues, which in turn would contribute to a higher proton concentration. The fact that degradation of pNHMM10 (with highest MAA content among pNHMMj hydrogels) was slowed in weak basic buffer supports the proposed autocatalytic mechanism.

Hydrogel degradation was largely decoupled from both stiffness and thermal transition behavior (Figure 4b), probably because the MAA content was too low to affect the

dominance of NIPAAm and highly hydrophobic MAPLA effects on thermal transition, and increases in electrostatic repulsion between MAA residues was not consequential. Theoretically, the ionizable MAA residues should increase pNHMMj copolymer hydrophilicity and pH sensitivity. Peppas et al. systematically studied the thermal sensitivity and pH sensitivity of poly(NIPAAm-co-MAA) hydrogels [32–34]. They found that the thermal transition temperature increased from 32°C to 34.5°C as MAA in the poly(NIPAAm-co-MAA) hydrogels increased from 0 to 12 mol%; on the other hand, the swelling ratio of the poly(NIPAAm-co-MAA) hydrogels increased significantly between pH 5.3 and pH 5.7 [33]. The pH sensitivity was also observed in a poly(NIPAAm-co-MAA) interpenetrating polymeric network [34]. In the current study, the examined pH range (6.4–7.4) was narrow, corresponding to infarcted cardiac muscle as a potential application area. The pH range studied fell above the pK_a of poly(methacrylic acid) as its effect on thermal transition behavior was studied for pNHMMj hydrogels containing small amounts of MAA. The pH sensitivity was not significant in the examined range and phase change and mechanical behavior that were attractive for minimally invasive delivery could be maintained, while the degradation rate could independently be optimized for a given application.

As expected, the pNHMM2 hydrogel showed faster *in vivo* degradation and disappearance from the site of injection compared to the other three hydrogels studied. However, the *in vivo* degradation and absorption of hydrogels qualitatively appeared to be substantially slower than *in vitro*. Day 21 was chosen as an endpoint for the *in vivo* studies based on the *in vitro* weight loss curve, expecting that almost all of the pNHMM2 hydrogel and about 50% of the mass of the pNHMM1 hydrogel would have been lost. Furthermore, hydrogel mass loss was expected to potentially be faster *in vivo* if local enzymatic activity contributed to the degradation process. The explanation for the apparently slower hydrogel loss *in vivo* could be that the catalyzing effect of MAA content was somewhat weakened *in vivo* as the tissue served as a more effective buffering system relative to the PBS *in vitro*.

The fluorescence of the remaining injected hydrogels was diminished for the faster degrading hydrogels with higher MAA content (Figure 9). This result was in accordance with what was observed *in vitro*. It is known that fluorescein changes from a lactone form to neutral and cation forms under low pH, and its emission intensity and quantum yield drop significantly at ~490 nm [35]. After 21 d in PBS, the fluorescence intensity of the labeled hydrogel interior dropped significantly for pNHMM0.5 and pNHMM1 versus that measured immediately after gelation, whereas the fluorescence intensity of pNHMM0 remained unchanged (Supplement Figure 4). This result would explain the relatively strong green fluorescence only being observed for the pNHMM0 hydrogel after 21 d *in vivo* (Figure 9), suggesting that the hydrogel interiors were more acidic for the other three studied hydrogels.

The foreign body response induced by the pNHMMj hydrogels 21 d post injection was characterized by local macrophage recruitment, as is typical of the foreign body response [36]. The macrophages mainly distributed around the hydrogel mass, forming an encompassing cell layer. Few foreign body giant cells could be observed. For the slow degrading pNHMM0, pNHMM0.5 and pNHMM1 hydrogels, the macrophages did not infiltrate or separate the hydrogel mass, as opposed to the findings with pNHMM2, where

the hydrogel was fragmented and the pieces surrounded by macrophages. This suggested that the in vivo absorption of pNHMMj hydrogels is not simply characterized by surface mass loss, but that degradation and solubilization occurs across the hydrogel with fragmentation. This fragmentation was observed in all cases in vitro, with fragmentation not occurring until the rapid phase of mass loss began.

The principle of manipulating acid autocatalysis may apply for controlling degradation rates for other thermally responsive hydrogels, and more broadly, biomaterials whose degradation is based on polyester hydrolysis. Ara et al. blended calcium compounds with different acidity with PLGA and found that the most basic calcium carbonate was most effective in delaying the degradation, while the most acidic calcium dihydrogenphosphate was least effective [37]. Similar buffering effects on autocatalysis were also found by Zhang et al., where basic magnesium carbonate and magnesium hydroxide significantly decreased the degradation rate of PLGA films while neutral sodium chloride and zinc sulfate did not show comparable influence [38]. Wu et al. have shown experimental results suggesting that pore morphology affects the diffusion of acidic degradation products from PLGA scaffolds, thus directly impacting the extent of acid catalyzed hydrolysis and degradation rate [39]. For pNHMM hydrogels, the mass loss curves were characterized by rapid degradation following early periods of slower change, similar to the behavior of several other polyester materials [40–42]. The autocatalytic mechanism might readily be applied to the design and synthesis for current material systems to introduce a tunable means of increasing degradation. For example, it is reasonable to assume that adding a small portion of pendant carboxyl groups onto PEG-PCL-PEG, PCL-PEG-PCL, or PEG-PLGA-PEG hydrogels could accelerate degradation, although such a design might also be dependent upon the overall hydrophilicity of the modified polymer system. Of note, a synthetic route to fabricate PCL-PEG-PCL copolymer bearing pendant carboxyl groups on the PCL blocks has been reported by Lavasanifar et al [43]. The degradation rate of thermally responsive hydrogels has been studied and utilized to control the release of pharmaceutical agents and proteins [44, 45]. The potential of thermally responsive hydrogels as cell carriers has been demonstrated as well [46–49]. Given that hydrogel degradability significantly influences cell proliferation, migration and differentiation [50–53], along with the therapeutic outcomes of injection therapies [54–56], adding acid to tune the degradability of thermally sensitive hydrogels is attractive.

One key advantage of the strategy employed here is that relatively small amounts of catalyst (acid) did not significantly influence the material properties of interest. However, there are potential limitations and drawbacks of this strategy. First, for the acid to play its role, a certain level of diffusion and access of protons to the ester bonds is needed, which in turn is dependent on the water content in the hydrogel. The accelerating effect may not be as readily achieved for more hydrophobic materials, particularly in the early stages of degradation. Also, for injectable hydrogels, the catalyzing effect may vary across injection sites under the influence of buffering capacity. Second, an acidic microenvironment would be generated for faster degradation to take place, which would also lower the pH for the cells and corresponding physiological events in the vicinity of the implant. This was not explicitly studied in this report, but the histological results did not suggest an obvious effect for the healthy skeletal muscle bed examined.

Conclusion

A series of NIPAAm based thermally responsive polymers (pNHMMj) were synthesized, which contained varying MAA content. By releasing protons that catalyzed ester hydrolysis, MAA accelerated the degradation and adsorption of the hydrogels both in vitro and in vivo, and this effect was positively correlated to MAA content in the copolymer. The thermal transition behavior and mechanical strength did not significantly vary among the pNHMMj hydrogels. On the other hand, the pNHMMj hydrogels showed low cytotoxicity and appeared to induce a mild foreign body response. The introduced autocatalysis strategy of tuning the degradation of thermally responsive hydrogels where degradation or solubilization is determined by their polyester components might be applied to other tissue engineering and regenerative medicine applications where appropriate biomaterial degradation behavior is needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) ^1H NMR spectra of pNHMM2. (b) MAA weight percentages in copolymers as determined by NMR and acid titration.

Figure 2.

(a) pH of supernatants of pNHMMj hydrogels after gelation. (b) Fluorescent emission intensity ratio between 540 nm and 440 nm of pNHMMj hydrogels mixed with LysoSensor pH-sensitive dye, excited at 360 nm. A higher ratio reflects a lower pH. Data of pNHMM5 and pNHMM10 were not available due to fast degradation. * and # indicate significant differences between and within groups, respectively.

Figure 3.

(a) Weight loss profiles of pNHMMj hydrogels. (b) Time for 50% weight loss derived from (a).

Figure 4.

Transition temperature of pNHMMj hydrogels. (a) Temperature sweep of shear modulus (G') of pNHMMj hydrogels. (b) Transition temperature dependence on pH.

Figure 5.
Young's modulus of pNHMMj hydrogels.

Figure 6. Viability of rSMCs encapsulated in pNHMMj hydrogels. (a–h) live rSMCs (red) stained with CellTracker in pNHMMj hydrogels 1 d and 7 d after encapsulation. Green: Fluorescein labeled hydrogels. (i) Percentage of live rSMCs after 1 d and 4 d encapsulation in pNHMMj hydrogels, determined by trypan blue staining.

Figure 7. Cytotoxicity of degradation products of pNHMMj hydrogels. rSMCs proliferation 1 d (a–e), 3 d (f–j), 7 d (k–o) after seeding determined by live/dead staining. (p) MTS assay of the rSMCs cultured in the same conditions.

Figure 8. Bright field and fluorescent images of excised rat leg muscles injected with pNHMMj hydrogels. Left column: excised on the same day of injection. Right column: excised 21 d after injection. The white mass and green fluorescence indicate the hydrogel.

Figure 9. Immunohistochemical (row (a,b)) and H&E (row (c)) staining of rat hindlimb muscle injected with pNHMMj hydrogels 21 d after injection. In row (a) and row (b): Blue: DAPI for nucleus, Green: hydrogels, Red: CD68 for macrophages. Images in row (b) are enlarged from corresponding areas in row (a), as indicated by yellow rectangles (may be rotated). M indicates muscle and G indicates hydrogel.

MAA feed ratio (%)	Feed ratio (%)			
	NIPAAm	HEMA	MAPLA	MAA
0	80	10	10	0
0.5	80	9.5	10	0.5
1	80	9	10	1
2	80	8	10	2
5	80	5	10	5
10	80	0	10	10

Scheme 1.

(a) Synthesis of poly(NIPAAm-co-HEMA-co-MAPLA-co-MAA). (b) Feed ratio of monomers.