Published in final edited form as:

J Biomed Mater Res A. 2009 September 15; 90(4): 1186–1197. doi:10.1002/jbm.a.32179.

In vivo evaluation of injectable thermo-sensitive polymer with time-dependent LCST

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Abstract

The focus of this study was to examine the biocompatibility, time-dependent LCST, and bioerodable properties of a copolymer system composed of NIPAAm, Dimethyl- γ -Butyrolactone (DMBL), and Acrylic Acid (AAc). Sprague Dawley rats were subcutaneously injected with 25 wt% solutions of Poly(NIPAAm-co-DMBL-co-AAc). At predetermined times, animals were sacrificed and polymer implants were recovered for characterization via $^1\text{H-NMR}$. In addition, polymer-contacting tissue sections were harvested and processed for histology. The biocompatibility of the implants was assessed by counting the number of fibroblasts and leukocytes present at the tissue-implant interface. The LCST data obtained from the *in vivo* implants was shown to agree with that of *in vitro* findings. Implant mass was shown to decrease after 4 days, indicating accelerated diffusion rates with increased implant swelling, hydrolytic degradation was confirmed with $^1\text{H-NMR}$ measurements. The cellular presence at the copolymer implant-tissue interface was shown to return to that of normal tissue 30 days post-implantation, which suggests a normal wound healing response.

Keywords

Hydrolysis; bioresorption; Thermally responsive material; hydrogel

1. Introduction

The potential use of injectable, *in situ*-forming polymeric systems for tissue engineering and drug delivery applications has sparked a great deal of research interest in recent years. Those polymeric systems that present temperature-sensitive, biodegradable characteristics are particularly well suited for these applications due to their ease of fabrication and relatively high water content, which contributes favorably to their biocompatibility. Some of these systems include poly(ethylene glycol)/poly(lactic acid-co-glycolic acid) (PEG/PLGA) triblock and graft polymers,^{1–2} triblock copolymers of methoxy poly(ethylene glycol) and poly(propylene fumarate),³ modified chitosan⁴, and poly(organophosphazenes) with poly(ethylene glycol) and amino acids.^{5–6}

The main utility of temperature sensitive polymeric systems for biomedical applications arises from the presence of a lower critical solution temperature (LCST). At temperatures below the LCST, the polymer is soluble in aqueous media, preferring an expanded or hydrated coil conformation; while at temperatures above the LCST, the polymer becomes insoluble, preferring a collapsed conformation.⁷ This reversible phase transition can occur over a narrow

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temperature range and is generally understood to be governed by the balance of hydrophilic and hydrophobic moieties on the polymer chains and the free energy of mixing.⁸ Such systems are particularly attractive for drug delivery applications as they can be engineered such that at temperatures below the LCST, a free-flowing combination of polymer and drug can be injected, which will subsequently gel at body temperature to form a solid polymer-drug implant.

Poly(N-isopropylacrylamide) (PNIPAAm) is a polymer that has an LCST of around 32°C, which makes it particularly attractive for drug delivery applications as it is soluble at ambient temperature and gels at physiological temperature of 37°C. The main drawback of PNIPAAm, however, is that it is not biodegradable. Ideally, an injectable drug delivery system would be biodegradable and clear itself through the kidney post delivery, thus eliminating the need for surgical removal. To overcome this drawback, several groups have investigated copolymers of PNIPAAm with hydrolysable and proteolytically degradable side groups. Kim et al. have reported on the synthesis of injectable poly(NIPAAm-co-acrylic acid) hydrogels with proteolytically degradable crosslinks.⁹ Neradovic et al. have studied several NIPAAm-based copolymers composed of various comonomers including 2-hydroxyethyl methacryl lactate (HEMA-lactate), N-(hydroxypropyl) methacrylamide lactate (HPMAm-lactate) and PEG, which were shown to possess time-dependant LCST properties.^{10–11} In addition, we reported the synthesis of copolymers of NIPAAm, HEMA-lactate, and acrylic acid that were shown to demonstrate time-dependant LCST properties.⁷

The presence of a time-dependant LCST imparts these systems with bioresorbable characteristics due to the degradation of side groups to produce a more hydrophilic polymer chain. This increase in chain hydrophilicity serves to increase the LCST of the remaining polymer. Thus, polymer systems with time-dependant LCST properties can be designed such that they can be dissolved in aqueous buffers at temperatures below the LCST, gel upon injection at 37°C, and re-dissolve, *in situ*, once the LCST has risen above 37°C, even though the backbone of copolymers does not degrade.

The synthesized copolymer of PNIPAAm and dimethyl- γ -butyrolactone (DMBL) was shown to possess bioerodible and time-dependent LCST characteristics. This material was also shown to possess limited cytotoxicity *in vitro*, with around 90% confluence after 15 days degradation.^{12–13}

The aim of this study was to show the *in vivo* biocompatibility of the poly(NIPAAm-co-DMBL-co-AAc). Additionally, this will allow for comparison of the biocompatibility of the material with the control provided by injections of poly(N-isopropylacrylamide).

2. Experimental

2.1. Materials

N-isopropylacrylamide (NIPAAm; Aldrich) was purified by recrystallization from n-hexane and vacuum-dried for four days. (R)-(+)- α -Acryloyloxy- β,β -dimethyl- γ -butyrolactone (DMBL; Aldrich) was used as received. 2,2'-Azobisisobutyronitrile (AIBN; Aldrich, 98%) was purified by recrystallization from methanol. Anhydrous 1,4-dioxane (Aldrich) was used as received. Other solvents used in this study were of reagent grade and used as received. Anhydrous Ethyl Ether was purchased from Sigma and used as received.

2.2. Synthesis of Poly(NIPAAm-co-DMBL-co-AAc)

Polymer synthesis was conducted following the reaction scheme shown in Fig 1. In brief, the NIPAAm/DMBL/AAc feed ratio was 91/7/2 mol.%, with a total monomer concentration of 0.1 g/ml in 1,4 Dioxane (10 wt%). AIBN was added in a ratio of 7×10^{-3} mole of initiator/mole of monomers. Two batches of approximately 10 g each were prepared. To reduce the

presence of oxygen during the polymerization reaction, nitrogen was bubbled through the solution at room temperature for 15 minutes prior to the addition of the initiator. The copolymerization reaction was conducted at 65°C for 16 h in a nitrogen atmosphere. After the reaction was complete, the polymer solution was filtered to remove impurities, upon which the polymer was precipitated in 10-fold excess diethyl ether, and chilled in an ice bath to encourage precipitation. The precipitate was recovered via filtration and vacuum-dried for 12 h. Polymer samples were then dissolved in distilled water at 5°C for 24 h and dialyzed (3,500 MW cutoff) at 5°C for three days. Dialysis water was changed once every 10–12 hours. After dialysis, the polymer was frozen overnight at –80°C and lyophilized for three days.

2.3. In vitro degradation

An *in vitro* degradation experiment designed to mimic the *in vivo* degradation was performed. Poly(NIPAAm-co-DMBL-co-AAc) (3 g) was mixed at 25 wt% with PBS (0.1 M, pH 7.4) and allowed to dissolve at 5°C for no less than 48 hours. Upon dissolution, 0.4 mL aliquots were loaded into plastic 3 cc syringes and injected into pre-weighed 18 ml glass vials. After injection, the polymers were allowed to gel at 37°C, at which time PBS (0.1 M, pH 7.4, 37°C) was added at a 10-fold excess. Samples were then incubated in a 37°C shaker at 50 rpm for the duration of the study. In order to simulate the continuous fluid exchange that takes place in the subcutaneous space, the PBS media was exchanged every day. At incubation times of 1, 4, 7, 10, and 14 days, polymer samples (n=3) were photographed and recovered for characterization. Sample recovery involved first removing the PBS supernatant and then dissolving the remaining polymer in distilled water at 5°C for 12 h. The polymer solution was dialyzed (3,500 MW cutoff) against distilled water at 5°C for approximately 8 hours. Dialysis water was changed every 2 hours throughout the 8 hour dialysis period. The dialyzed solution was recovered, frozen overnight at –80°C, and subsequently lyophilized for 3 days.

2.4. In vivo degradation and biocompatibility

2.4.1. Injection Preparation—Prior to preparing the injection solution, the lyophilized polymers were sterilized via ethylene oxide (EtO) gas sterilization. Upon sterilization, polymers were mixed at 25 wt% with sterile PBS (0.1 M, pH 7.4) in a sterile 50 ml centrifuge tube and allowed to dissolve at 5°C for 48 hours. Upon dissolution, 0.4 ml aliquots were loaded into sterile, 3 ml luer-lock syringes, which were then capped with 18G needles. Negative control injections of PNIPAAm (25 wt%) homopolymer were also prepared via the same procedure. All material handling prior to injection was performed in a clean hood under sterile conditions. In order to keep the polymers from precipitating, all loaded syringes were kept at 5°C until ready to inject.

2.4.2. Injection Procedure—Male Sprague Dawley rats (~200 g) were used for the *in vivo* biocompatibility and degradation studies. Prior to injection, rats were anesthetized using a cocktail of ketamine (50 mg/ml), xylazine (5 mg/ml), and acepromazine (1 mg/ml). Each rat was injected with 0.1 ml of cocktail per 100 g body weight. Upon anesthetization, the back of each animal was shaved and 0.4 ml injections of the appropriate polymer solution (25 wt%) were administered subcutaneously. In addition to Poly(NIPAAm-co-DMBL-co-AAc) injections, PNIPAAm homopolymer injections were administered as the control for the biocompatibility study. In an effort to capture any animal-to-animal variability in the cellular immune response, animals in the biocompatibility study received both Poly(NIPAAm-co-DMBL-co-AAc) (n=3) and Poly(NIPAAm) (n=1) injections. The animal study was approved by Arizona State University IACUC, and the animals were housed under conditions that met IACUC requirements; NIH guidelines for the care and use of laboratory animals (NIH Publication #85–23 Rev. 1985) have been observed.

2.5. Sample Harvesting

2.5.1. Biocompatibility Study—At time points of 1, 7, 14, and 30 days post-implantation, two animals containing both Poly(NIPAAm-co-DMBL-co-AAc) and PNIPAAm injections were sacrificed by CO₂ asphyxiation. Each implant was then surgically exposed, photographed, and removed along with the implant-contacting dermal tissue. Excised tissue sections were then placed in individual processing cassettes and fixed in 10% neutral buffered formalin for at least 24 hours at room temperature prior to processing. The sections were embedded in paraffin wax and sectioned at a thickness of 5 μ m. For each recovered sample, two transverse sections were taken at points to the right, left and along the midline of each implant, for a total of 6 samples.

2.5.2. Degradation Study—For the degradation study, animals with implant procedures similar to the in vivo biocompatibility study above were sacrificed at time points of 1, 4, 7, 10, and 14 days post-implantation. Upon sacrifice, the implants (n=3) were surgically exposed by carefully dissecting the tissue around the implant. Each implant was then photographed and carefully removed. Upon removal, the recovered polymers, along with minimal amounts of residual tissue, were immediately placed in 5 ml of 0.3M NaF solution at 5°C and allowed to dissolve. The solution was then filtered to remove residual tissue and impurities. Further purification was conducted as follows: The filtered product was then placed in 50 ml centrifuge tubes and immersed in a 37°C bath to induce precipitation of the polymer. The presence of NaF decreased the solution LCST leading to more rapid precipitation upon immersion in the 37°C bath, thus excluding any residual proteins left in solution from the precipitated polymer. To further isolate the polymer, the mixture was centrifuged at 1000 rpm for 60s. After centrifugation, the supernatant was removed and distilled water (10-fold excess) was added. The polymer was then allowed to dissolve at 5°C for approximately 12 h. Once polymer dissolution was complete, residual salts were removed via dialysis (3,500 MW cutoff) against distilled water at 5°C for 8 h. The dialysis water was changed every 2 h over the 8 h dialysis period. The remaining polymer solution was then frozen overnight at -80°C and lyophilized.

2.6. Sample Characterization

2.6.1. Histology—All tissue was sent to La Trish Fialson at the Medical College of Georgia for sectioning and staining. Initially, sections were stained with Hematoxylin and Eosin (H&E). In addition to H&E staining, sections were also stained with Masson's Trichrome and Giemsa stains to evaluate the collagen deposition and lymphocyte activation, respectively, at the tissue-implant interface. Tissue sections were examined for leukocyte and fibroblast activity using light microscopy (Leica, DM IRB). Micrographs (n=6), at 100 $\times$ total magnification were taken of tissue cross-sections from each time period. These micrographs were taken at sites to the left, right, and along the midline of the implant in order to capture any variability in cell quantity that may be present at different sites around the implant. The number of dark staining cells in H&E sections, which were assumed to be leukocytes and fibroblasts, were then counted using masking techniques to resolve darker cell bodies from lighter background tissue. Lymphocytes are generally understood to have a cell diameter of 8 to 12 μ m, and fibroblasts nuclei are similarly sized. A cell area of 50 μ m², which corresponds to a cell diameter of around 8 μ m, was used to resolve clusters of cells. In an effort to assess the specificity of the cellular response to the implant interface, counts were also performed at 400X total magnification at varying distances from the tissue-implant interface. In addition to the H&E stained sections, cell counts were performed on Giemsa stained sections to determine lymphocyte activity. Masson's trichrome sections were also examined to evaluate the degree of collagen deposition at the implant interface. Cell counts (n=6, per time period) for Poly(NIPAAm-co-DMBL-co-AAc) and Poly(NIPAAm) implants are reported as mean cell count $\pm$ standard deviation.

2.6.2. Turbidity Measurements—The LCST of the degraded copolymers was determined via turbidity measurement (cloud point determination). Copolymer samples were dissolved in PBS (0.1 M, pH 7.4) at a concentration of 1 wt%. After dissolution the copolymer solution was adjusted to pH 7.4 using 1 M NaOH. The turbidity of copolymer solutions was determined via transmittance measurements using spectroscopy. The transmittance of the copolymer solutions at a fixed wavelength of 500 nm was recorded as a function of temperature (heating rate: 1°C min⁻¹). The LCST of each respective sample was interpreted as the temperature at which the solution transmittance reached 50%. Turbidity measurements for the *in vitro* and *in vivo* degraded Poly(NIPAAm-co-DMBL-co-AAc) copolymers were performed in triplicate for each time period. LCST data is reported as the mean ± standard deviation for these runs.

2.6.3. ¹H-Nuclear Magnetic Resonance—¹H-Nuclear Magnetic Resonance (¹H-NMR) was used to determine the degree of hydrolysis in the *in vitro* and *in vivo* degraded copolymers. Measurements were performed using a Varian Gemini-300 spectrometer operating at 300 MHz in the Fourier-transform mode. Copolymer samples were dissolved in deuterated water (D₂O) at a concentration of 7 wt% prior to ¹H-NMR analysis.

2.7. Statistics

Statistical significance was determined using the two sample t-test, with cell counts from normal tissue (no injection) serving as the base mean. Data was deemed significant for p<0.05.

3. Results

3.1 Synthesis of Poly(NIPAAm-co-DMBL-co-AAc)

Table 1 provides the summary characteristics of the synthesized polymer. Briefly, the polymer showed a 6.4 % DMBL incorporation, and a 2.0 % AAC incorporation. The final polymer had a number average molecular weight of approximately 0.8 × 10⁵ g/mol, and a PDI of 2.1. The initial LCST was determined to be 17.4 °C

3.2. In vitro degradation

Figure 2 shows the condition of the polymer gel at various stages of incubation. Fig. 2(a) was taken immediately after the polymer was injected into a 37°C environment, and represents the physical cross-linking of polymer chains induced by increasing the environmental temperature above the LCST of the polymer. The swelling pattern shown in Figure 2 is consistent with the hydrogel swelling dynamics previously described by Peppas.¹⁴ This swelling pattern is characterized by the presence of an inward-moving, radial swelling front, forming an interface between the relatively water-rich gel phase, and the shrunken precipitate core, seen in Fig. 2 (b). The presence of the precipitate core limits swelling to two dimensions during the early stages of swelling due to the formation of a compressive stress on the precipitate phase, which is caused by the advancing swelling front, and a resultant tensile stress on the gel phase. Once the swelling front(s) meet at the center, which occurs after around 4 days incubation, the precipitate core vanishes, thus enabling swelling to occur in three dimensions. After 7 days, Fig. 2(c), the polymer gel clearly begins to disintegrate, and, as Fig. 2(d) shows, has become completely dispersed in solution after 14 days. In addition to swelling alone, dissolution is due to the hydrolysis of ester bonds within the dimethyl-γ-butyrolactone (DMBL) ring, causing an increase in the LCST of the polymer chains.

Evidence of this phenomenon is seen in Fig. 3, which shows representative turbidity measurements for polymer-PBS solutions for varying times of *in vitro* degradation. The LCST for each polymer sample was obtained from the turbidity curves and was interpreted as the temperature at which the transmittance of light decreases to 50%. The data show an increase in LCST as a function of degradation time, with a final LCST of 36°C after 14 days incubation.

It is important to note that these turbidity measurements were obtained from recovered material, and thus do not take into account the contributions of degraded polymer chains that have diffused out of the matrix into the bulk media. In order to simulate the fluid exchange that occurs in the subcutaneous space, the PBS media was exchanged each day for the duration of the in vitro study. Thus, any chains that had undergone sufficient hydrolysis to achieve an LCST above 37°C likely diffused out of the polymer matrix and were removed during media exchange. Thus, while the average LCST of the recovered material after 14 days incubation is just below body temperature, a significant fraction of the original polymer chains had achieved a LCST of above 37°C by this time, as evidenced by the dissolution of the gel seen in Fig. 2 (d).

3.2. In vivo degradation

The swelling behavior of the material in vivo was found to generally agree with in vitro findings. Fig. 4 is a collection of the implant photographs taken prior to sample recovery and shows the dynamic swelling behavior and eventual dissolution of the polymer implants. After 1 day, as shown in Fig. 4(a), the implant closely resembles that which was seen in the in vitro study; displaying a precipitate core interfacing with an advancing swelling front. Moderate swelling is seen after 4 days, as seen in Fig. 4(b), with more dramatic swelling noticeable by 7 days, Fig. 4(c), post-implantation. The amount of swelling seen after 7 days represents the maximum observed swollen volume, as the 10 day implants (not shown) were approximately the same volume. This also agrees with in vitro findings as the polymer gels appeared to be at their most swollen state after 7 days incubation. While the in vitro samples were shown to take on a hemispherical geometry upon swelling, the implants clearly swell to a more spherical geometry in vivo. Swelling of the in vitro samples was constrained due to the presence of the sample container, while the in vivo implants were constrained equally in all directions, and thus exhibit equal swelling in all directions upon dissolution of the precipitate core.

The photo shown in Fig. 4(d) was taken at 14 days after post-implantation and shows that total dissolution of the implant occurs somewhere between 10+ to 14 days after post-implantation, which also agrees with in vitro findings. The implants also seem to be somewhat angiogenic, due to a foreign body response, as Fig. 4(b) shows the onset of blood vessel formation after 4 days. The degree of angiogenesis is more pronounced after 7 days, with the clear formation of a highly branched vessel system shown in Fig. 4(c). After complete polymer resorption, however, the tissue, shown in Fig. 4(d), only displays minimal irritation.

After the polymer implants were photographed, they were excised, purified, and characterized to determine the time-dependant mass loss and LCST properties. Fig. 5 shows a plot of recovered polymer mass as a function of implant time, and representative turbidity curves showing the change in LCST with time. Fig. 5(a) shows that implant mass-loss seems to begin after 4 days implantation. This agrees with in vitro findings where it was determined that during the early stages (from 0–4 days) of degradation, the rate of swelling is constrained due to the presence of the precipitate polymer core, which limits the degree of water penetration of the polymer matrix. This constraint limits the swelling ratio of the gel, and thus limits its achievable volume. This lack of adequate volume, coupled with the increased tortuosity associated with diffusing around collapsed polymer chains, serves to decrease the diffusion rate of soluble chains from the polymer matrix during this time. The dissolution of the precipitate core, which occurs from 1 to 4 days, releases the swelling constraint, resulting in increased swelling from 4 to 7 days. This increased swelling causes an increase of implant volume, which permits the increased rate of diffusion of soluble polymer chains from the implant matrix as seen in Fig. 5(a). The turbidity curves as shown in Fig. 5(b) show a trend similar to that of the in vitro samples, showing both an increase in LCST with degradation time, and an increase in the temperature range over which the solution changes from transparent to turbid. Both of these

characteristics indicate an increased presence of hydrophilic chains with degradation time. This phenomenon is more clearly seen in Fig. 6, which displays the time-dependant LCST properties of the material for both the in vitro and in vivo degradation studies. Fig. 6(a) shows very little difference in LCST between the in vitro and in vivo degradation studies, which indicates that hydrolysis is the predominant mode of degradation in vivo. In addition, the temperature range of turbidity transition, shown in Fig. 6(b), for the in vivo implants shows a similar pattern to that of the in vitro samples. The range of turbidity transition gives an indication of the breadth of the LCST distribution within the polymer sample. In each case, the range of transition increased dramatically after 7 days, indicating an increased rate of hydrolysis during this time. This is to be expected as the maximum swollen volume of the gels was achieved between 7 and 10 days degradation. The increased water content of the gels during this time would increase the rate of hydrolysis, and thus the heterogeneity of the polymer chains within the matrix, causing a widening of the LCST distribution.

Further evidence of time-dependant hydrolysis is seen in Fig. 7, which shows the ^1H -NMR spectra for both in vitro and in vivo degraded Poly(NIPAAm-co-DMBL-co-AAc) samples at various stages of degradation. The methylene (2H) and methine(1H) peaks appear at 4.2 (•) and 5.5 (→) ppm. Fig. 7 shows a gradual dampening of the DMBL characteristic peaks with degradation time in both the in vitro and in vivo samples, which seems to occur after 10 days degradation in each case. At 10 days, the emergence of a new peak(2H) at around 3.4 ppm can also be seen even though the methine peak (1H) is hard to see owing to partial overlap with H₂O. As the hydrolysis scheme shown in Fig. 7 points out, hydrolysis of the DMBL ester bonds results in the opening of the butyrolactone ring which yields a polymer chain containing more hydrophilic moieties (carboxylic acids and hydroxyl groups). This ring opening causes a shift of the DMBL characteristic peaks in the ^1H -NMR spectra. It is this chemical shift in response to the opening of the butyrolactone ring that causes both the dampening of the DMBL characteristic peaks, and the appearance of peaks at 3.4 and 4.6 ppm, seen in the spectrum of the 14 day in vitro degraded sample. In our previous paper, it was confirmed by FT-IR and FT-NMR that the butyrolactone ring is opened by hydrolysis and that the ester attached to the backbone is stable at pH 7.4 at 70 °C.¹³ Thus, it seems clear from the spectra shown in Fig. 7 that hydrolysis is indeed taking place and that both the in vitro and in vivo degraded polymers seem to exhibit a similar hydrolysis pattern.

3.3. In vivo biocompatibility

At times of 1, 7, 14 and 30 days post-implantation, animals were sacrificed and the dermal tissue sections surrounding the implant (or implant vacancy) were harvested and processed for histology. Specimens were stained with Hematoxylin and Eosin (H&E) and analyzed using light microscopy. The severity of the foreign body response was quantified by counting the number of leukocytes and fibroblasts present within the connective tissue adjacent to the implant. Fig. 8 shows a graphical representation of the location of cell counts relative to the implant pocket. In routinely stained H&E sections, the nuclei of leukocytes and fibroblasts stain a dark purple and are thus easily differentiated from the surrounding acellular connective tissue. In order to adequately gauge the response of the polymer, tissue sections from control animals were also studied. Control animals consisted of those animals receiving no injection (baseline), and those that received injections of Poly(NIPAAm) homopolymer (control). Fig. 9 shows micrographs of (a) native tissue, and of the experimental polymer implants after (b) 1 day, (c) 1 week, and (d) 1 month implantation. The micrographs show a clear incidence of leukocyte and fibroblast (dark staining cells) hyperplasia in response to the Poly(NIPAAm-co-DMBL-co-AAc) implants at both 1 and 7 days post-implantation. This response, however, seems to diminish with time as the cell population after 1 month closely resembles that of native tissue. The positions marked with an asterisk (*) in Fig. 9 indicate the interface between the polymer implant and surrounding connective tissue. It is interesting to note that the increase

in cellular activity does not seem to be limited to the area around the implant interface alone, but rather seems to consist of a random distribution of cells throughout the connective tissue surrounding the implant. The wound healing response consists of several overlapping phases, which include the inflammation phase, the proliferative and repair phase, and the remodeling phase,¹⁵ all of which contribute to tissue repair. The inflammation phase is histologically characterized by an increase in inflammatory leukocytes at the site of injury in response to the release of chemoattractant growth factors from platelets, activated leukocytes, and other cell types. Histological evidence of inflammation can typically be seen within 1–2 days of injury, which may explain the increase in cell number seen in Fig. 9(b). Following migration of inflammatory leukocytes (neutrophils and monocytes) to the site of injury, these cells in turn secrete more growth factors which cause a rapid migration and proliferation of several cell types including neutrophils, macrophages, fibroblasts, endothelial, and others. This represents the initiation of the proliferative and repair phase of wound healing, and may explain the noticeable increase in cell number seen after 7 days implantation. The repair phase often occurs in concert with the proliferative phase and is initiated by the formation of extracellular matrix around the implant area, which is accomplished primarily by fibroblasts. After extracellular matrix has been formed, neovascularization occurs followed by the formation of granulation tissue. The histologic indicators of granulation tissue development are the formation of numerous capillaries and the increased presence of fibroblasts in the vicinity of the wound. Fig. 9(c) shows both an increased cellular presence and the formation of numerous capillaries in the vicinity of the tissue-implant interface, which may indicate the formation of granulation tissue after 7 days. It is generally understood that the formation of granulation tissue is histologic evidence of normal resolution of the inflammatory phase of the wound healing process.¹⁵ During the later stages of tissue repair, the cellular presence around the wound area decreases and granulation tissue is gradually replaced by acellular scar tissue. This process seems to be taking place after 30 days, Fig. 9(d), as evidenced by the significant decrease in cellularity at the tissue-implant interface.

In an effort to quantify the degree of cellular response to both the experimental and control implants, the cell number at several locations ($n=6$) around each implant was determined using image analysis software. Of primary interest was the presence of leukocytes and fibroblasts, which represent the dark-staining cells in H&E stained connective tissue sections. Fig. 10 shows the cell count as a function of implant time for each of the implant materials tested. The data show a significant increase ($p<0.05$) in cellular response to the Poly(NIPAAm-co-DMBL-co-AAc) implants from initial implantation to 2 weeks degradation, while little change in cellular activity was observed for the Poly(NIPAAm) implants over time. The increased cellular response to the Poly(NIPAAm-co-DMBL-co-AAc) implants reaches its maximum at 1 week post-implantation. The degree of cellular response to the Poly(NIPAAm-co-DMBL-co-AAc) implants returns to that of native tissue at 30 days post-implantation.

The degree of collagen deposition at the tissue-implant interface was qualitatively investigated by examining sections with Masson's trichrome. Fig. 11 shows an increased presence of collagen deposition around the implant site, with maximum collagen presence after two weeks. It does appear that collagen density decreases significantly by 30 days; however the collagen density at this time is still above that of normal tissue. Further, the collagen fibers appear to be more ordered in the area around the implant, which differs from the irregular morphology of the collagen fibers in normal tissue. This presence of an increased density of ordered collagen fibers indicates the presence of residual scar tissue after 30 days implantation.

4. Conclusions

This study aimed to investigate the degradation, time-dependant LCST, and immunogenic properties of a novel in situ-forming hydrogel for drug delivery applications. In vitro

degradation studies showed that the material exhibited both time-dependant LCST and erodible properties. This material has been shown to be reasonably biocompatible, with little tissue irritation remaining after an initial wound healing phase. While there is evidence of capsule formation around the implant, further study will be necessary to determine if this capsule meaningfully inhibits drug release from the polymer system.

Acknowledgments

The authors would gratefully like to acknowledge funding from the NIH grant GM065917. We would also like to acknowledge the skillful and professional histological services provided by the Medical College of Georgia.

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Fig. 1.
Synthesis scheme of Poly(NIPAAm-co-DMBL-co-AAc).

(a)

(b)

(c)

(d)

Fig. 2.
In vitro swelling of Poly(NIPAAm-co-DMBL-co-AAc) after (a) 0 days; (b) 1 day; (c) 7 days;
(d) 14 days incubation at 37°C in PBS (0.1 M, pH 7.4)

Fig. 3. Turbidity curves for in vitro degraded Poly(NIPAAm-co-DMBL-co-AAc) gels. The curves represent the transmittance of UV light (500 nm) as a function of temperature for samples incubated at 37 °C for (X) 1 day, (▲) 4 days, (■) 7 days, (◆) 10 days and (○) 14 days.

Fig. 4. Poly(NIPAAm-co-DMBL-co-AAc) implants (a) 1 day; (b) 4 days; (c) 7 days; (d) 14 days post-implantation. All implants were completely eroded after 14 days

Fig. 5.

(a) Dry weight of recovered Poly(NIPAAm-co-DMBL-co-AAc) implants as a function of implant time (n=3). (b) Representative turbidity curves for Poly(NIPAAm-co-DMBL-co-AAc) samples after (X) 1 day, (▲) 4 days, (■) 7 days, and (◆) 10 days in vivo degradation

Fig. 6. (a) Mean LCST as a function of degradation time for (◆) in vitro and (■) in vivo degraded Poly(NIPAAm-co-DMBL-co-AAc) samples (n=3); (b) Plot of the temperature range of transition as a function of degradation time for (◆) in vitro and (■) in vivo turbidity measurements (n=3)

Fig. 7. (Top Panel) Hydrolysis mechanism of Poly(NIPAAm-co-DMBL-co-AAc) showing the opening of the butyrolactone ring upon hydrolysis. (Lower Panel) ¹H-NMR spectra showing a hydrolysis-dependant shift of the butyrolactone characteristic peaks for both in vitro (left) and in vivo (right) degradation.

Fig. 8.
Graphic representation demonstrating the location of 100× cell counts in relation to the implant pocket

Fig. 9. Histological micrographs of (a) native tissue, and of the connective tissue surrounding Poly (NIPAAm-co-DMBL-co-AAc) implants after (b) 1 day; (c) 7 days; (d) 30 days implantation. The positions marked with an asterisk (*) correspond to the location of the tissue-implant interface.

Fig. 10.

Mean cell count for each material tested. Counts were taken at 100 $\times$ total magnification at varying locations within the connective tissue immediately surrounding the implant site. Data shown for Native tissue (diagonal fill); Poly(NIPAAm-co-DMBL-co-AAc) (solid fill); Poly (NIPAAm) homopolymer (horizontal fill). (n=6)

Fig. 11. Tissue sections stained with Masson's Trichrome to reveal collagen density. Sections were taken from (a) native tissue, and adjacent to Poly(NIPAAm-co-DMBL-co-AAc) implants after (b) 1 day, (c) 14 days, and (d) 30 days. The asterisk (*) represents the location of the tissue-implant interface

Table 1

Initial characterization for synthesized poly(NIPAAm-co-DMBL-co-AAc)

NIPAAm	Composition (mol %)		M_n ($\times 10^5$ /mol)	M_w/M_n	Cloud point($^{\circ}$ C)
	DMBL	AAc			
91.6	6.4	2.0 $\pm$ 0.1	0.8	2.1	17.4

Table 2

Injection regimen for the in vivo biocompatibility and degradation studies.

study	polymer	# of injection	# of animals
biocompatibility	Poly(NIPAAm-co-DMBL-co-AAc)	24	8
	Poly(NIPAAm)	8	
degradation	Poly(NIPAAm-co-DMBL-co-AAc)	30	10